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Smoking, nicotine and the kidney

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To my family

Paranimfen: Saritha Adepu
Tushar Tomar

Contents

Chapter 1

General Introduction

Chapter 1

Chronic kidney disease and end stage renal disease

Kidneys in all the vertebrates play a central role in overall homeostasis of the body. Main function of the kidney is to purify blood via glomerular filtration and excrete waste products in urine. Kidneys also play an eminent role in maintaining blood pressure, acid-base homeostasis, electrolyte balance and have a variety of endocrine functions, such as the production and secretion of erythropoietin for regulation of blood hemoglobin levels.

Many different direct or indirect insults can lead to chronic kidney disease (CKD), which over time may progress to end stage renal disease (ESRD). ESRD is the terminal stage of kidney disease. The common clinical signs of progression towards ESRD are a progressive decline in glomerular filtration and abnormal glomerular protein leakage (1). Proteinuria is not only a marker of renal damage but also a mediator of the progression of renal disease. In overtly proteinuric conditions, the non-physiological presence of proteins activates tubular epithelial cells. Activated tubular cells induce pro-fibrotic- and pro-inflammatory signaling and cells, which lead to tubulo-interstitial damage and progression of CKD (2-4). Once kidneys fail, renal replacement therapy (RRT) is necessary for survival of patients. Dialysis and kidney transplantation are the two available forms of RRT. If feasible, kidney transplantation is preferred over dialysis in terms of survival and quality of life (5,6).

Chronic transplant dysfunction

The first renal transplantation was successfully performed in 1954 in identical twins, in which no immunological barriers exist. After that, it took many years to find immunosuppressive regimens that allowed for performance of renal transplantation between donors and recipients that are not genetically identical. Since then, much improvement has been achieved in both short and long term graft survival (7). However, the pace of improvement in long term graft survival is strongly lagging behind the improvements in short term graft survival (8). The main causes of late graft loss are death with a functioning graft and chronic transplant dysfunction (CTD). CTD is defined as progressive decline in kidney function in the presence of high blood pressure and proteinuria, typically occurring beyond the first 3 months after transplantation. The histological hallmarks of CTD are glomerulosclerosis, transplant arteriosclerosis and interstitial fibrosis with tubular atrophy (9). Transplant arteriosclerosis is characterized by neo-intima formation within the arterial vasculature in the transplanted organ, that leads to thickening and hardening of vessels, that in severe cases can ultimately lead to occlusion of the vessel lumen (10). The progressive luminal occlusion leads to reduced blood supply and ischemic injury. The etiology of CTD is not clear. It is, however, recognized to be a multi-factorial process. Both immunological and non-immunological factors contribute to the development of CTD (11). The major known risk factors of CTD are summarized in table 1, by a break-up in immunological and non-immunological risk factors. It is noteworthy that many of the non-immunological risk factors for CTD are also cardiovascular risk factors, such as hypertension, dyslipidemia, diabetes etc. Smoking is also a very well-established cardiovascular risk factor, but its role in CTD has not been well-established. In this thesis, we investigate the hypothesis that smoking is an important additional risk factor for CTD.

Table 1: Major risk factors associated with chronic transplant dysfunction

Immunological risk factors	Non-immunological risk factors
HLA mismatch	Recipient race, age and sex
Suboptimal immunosuppression	Donor age, sex, health status
Repeat transplantation	Duration of dialysis
Acute rejection episodes	Ischemia reperfusion injury
Anti-donor antibodies	Proteinuria
	Dyslipidemia
	High BMI
	Hypertension
	Diabetes

CMV infection
Treatment non-compliance

Smoking

Smoking: past, present and future

Smoking of tobacco is a very old practice. Rodrigo de Jerez and Luis de Torres first introduced tobacco to Europe in 1492 and it is in use ever since. Only recently, adverse health effects of smoking were recognized and lead to strong anti-smoking awareness. As a consequence, the prevalence of smoking in the USA general population declined from 42% in 1965 to 25 % in 2000. Despite these falling trends, many people are still smoking (e.g. 46 million still continue smoking in the USA alone). This poses them at substantial risk for cardiovascular and chronic diseases in 2005 (12). Currently the prevalence of smoking in the world varies between 40 and 6 %. In 2010 The Global Tobacco Survey was done in China. It was found that 28.1% of Chinese population was smoker with a huge male dominance, as exemplified by a male to female ratio of 22:1 (13). Smoking is highly addictive and therefore it is very difficult to quit smoking. However public policies are made widely to discourage the use of cigarette smoking. Many initiatives are underway. In England for example, the smoking toolkit study (STS) has been designed to evaluate longitudinal trends in cigarette smoking cessation. The first data from this study are expected by 2014 (14).

Smoking and chronic diseases

Smoking is an acknowledged risk factor for different forms of cancer and several chronic diseases. Among cancer, strongest associations exist with lung and bladder cancer (15-17). Smokers have a 20 times higher risk of lung cancer than non-smokers (18). Among chronic diseases, the strongest association of smoking exists with chronic obstructive pulmonary disease (COPD), a major form of chronic lung disease. Smokers have a 25 times higher risk for COPD than non-smokers (19,20). Smoking is also an established risk factor for cardiovascular disease (21-23). In the large INTERHEART study, smokers had an odds ratio of 2.87 for myocardial infarction compared to non-smokers (24).

Smoking as a risk factor for kidney diseases

Smoking has recently been identified as a renal risk factor as well. Smoking is found to be associated with kidney injury both in the general population and in the patients with elevated risk for kidney disease. In a cross-sectional analysis of the Prevention of REnal and Vascular ENd stage Disease (PREVEND) study, it was found that smoking is associated with albuminuria in the general population (25). It has also been shown that smoking is associated with albuminuria in hypertensive patients (26). Data from the Multiple Risk Factor Intervention Trial (MRFIT) showed a dose dependent relation between cigarette smoking and risk of development of ESRD in the general population (27). Furthermore, in patients with diabetic nephropathy it has been shown that kidney disease progresses twice as fast in smokers as in non-smoking patients (28). Likewise, smoking has been shown to be a risk factor for progression of IgA nephropathy (29-33).

Interestingly, all these observations involve native kidney diseases, in which the kidney is fully innervated. One of the contemporary theories is that the detrimental effects of smoking on kidneys is through the action of nicotine on the sympathetic nervous system, thereby inducing intrarenal vasoconstriction and parenchymal ischemia (30,34,35). It should be noted that the kidneys of renal transplant recipients are denervated. As such, they could be protected from the adverse effects of smoking by this hypothetical pathway. So far, no studies have been performed in RTR. In **chapter 2** of this thesis we investigated the risk of mortality and graft failure associated with smoking in a stable renal transplant cohort.

Another population of interest in this perspective is that of lung transplant recipients. Progressive renal damage is common in this population with a major role of CNI required for lung graft survival. Also, early per- and peri-operative hemodynamic instability is a likely contributing factor. As smoking is a main progression factor in chronic pulmonary disorders, many patients that require a lung transplant have a history of heavy smoking. However, they have to stop smoking before being considered for transplantation. This population is therefore suitable to investigate the effects of former smoking in terms of mechanisms. Activation of renal nerves could at first lead to a hemodynamically mediated decline in renal function due to vasoconstriction, but eventually cause tubulo-interstitial injury due to chronic ischemia. Therefore if a current vasoconstrictive effect of nicotine is the main cause of the association of smoking with impaired renal function, one would not expect an association of former smoking with chronic kidney disease these patients suffer from. If, however, the effect is mediated by other toxic effects, former smoking could still be associated with renal disease in these patients. In **chapter 3** of this thesis we describe the investigations that we performed on this topic in the lung transplant cohort of our hospital.

Alcohol consumption and mortality

In our studies on smoking in RTR we found a positive association of smoking with alcohol consumption. This is an association that has also been found in other populations (36). Along with cigarette smoking alcohol is a usual part of social gatherings, dinner and parties. Excessive intake of alcohol is called alcoholism and leads to conditions like malnutrition, laryngeal cancer, esophagus cancer, depression, liver cirrhosis and liver cancer (37-42). Mild alcohol intake, however, is known to be beneficial for survival in the general population (43,44). In the general population, alcohol usage and mortality show a J shaped curve, with mild users protected compared to non-users and heavy users (45). Also, the relation of alcohol consumption with cardiovascular disease has been widely studied. A Danish study showed an inverse association between alcohol consumption and risk of coronary artery disease (46). They showed that in moderately drinking men, risk of coronary artery disease was reduced by about 25% compared to non-drinkers. Little is known about alcohol consumption in renal transplant recipients. One study found an increased risk of graft failure in recipients with alcohol addiction pre-transplantation (47). One other study reported on the prevalence of social alcohol consumption post-transplantation (48), but to the best of our knowledge no studies exist on a potential association of post-transplantation alcohol consumption with graft failure or mortality. In **chapter 4** of this thesis we investigated the association of smoking with alcohol consumption in RTR on one hand and the influence of smoking and alcohol consumption on graft failure and all-cause mortality on the other hand.

Nicotine

In the second part of this thesis, we aimed to more closely investigate the effect of nicotine on CKD. Nicotine is one of the best known and the most abundant component of cigarette smoke. Nicotine is obtained from tobacco and highly addictive in nature. Once inhaled or taken in oral form as snuffs or chewing gum or used in the form of dermal patch, nicotine rapidly reaches the liver and the brain. Nicotine binds to the nicotinic acetylcholine receptors (nAchR) (49,50). To date, 17 nAchRs have been described. Nicotine binds to various subtypes of nicotinic acetylcholine receptors present in central and peripheral nervous system.(51)The addictive nature of nicotine is due to immediate reward from the brain dopaminergic pathways (52,53). Nicotine is metabolized by cytochrome P450 enzymes of the liver into various metabolites. Nicotine and its metabolites are excreted from the body in urine, in stool or by exhalation (54).

In humans nicotine replacement therapy (NRT) forms the first line of therapy to support smoking cessation and to reduce the withdrawal symptoms of quitting smoking. NRT is a controlled way of delivering nicotine to break the reward cycle from nicotine in cigarette smoke. Randomized control trials have shown that all forms of NRT have added beneficial effects for long-term abstinence from cigarette smoking.

Nicotine and inflammation

Recent literature showed that nicotine possess anti-inflammatory properties (55-57). Nicotinic receptors are also present on monocytes and endothelial cells. Activation of these cells is crucial for sustained inflammation. The anti-inflammatory effects are largely mediated through a7-nAchR. Binding of nicotine to the a7nAchR present on endothelial and macrophages leads to deactivation of those cells and thereby down-regulation of inflammation. In line with these actions, nicotine has been found to be associated with a more favorable prognosis in many observational studies of diseases of inflammatory origin, e.g. Ulcerative Colitis, pneumonitis, and arthritis (58). In animal models nicotine attenuates ischemia-reperfusion-induced kidney damage (59-61). Thus, whereas nicotine has been suggested to be a “nephrotoxic” component of cigarette smoke by inducing intra-renal vasoconstriction and renal ischemia (62,63), it could also have renal protective properties by ameliorating intra-renal inflammation, as the latter is assumed to be involved in progression of CKD, as outlined below.

Proteinuria and kidney inflammation

Intrarenal inflammation is involved in many, if not all types of progressive renal damage. In some renal conditions an inflammatory disorder is the primary trigger for renal damage, such as ischemia reperfusion injury, or glomerulonephritis. However, in many CKD patients no such specific primary disorder is present, but nevertheless inflammation is relevant to progressive renal function loss. As stated above, proteinuria is a main clinical risk factor for progression of CKD, by a sequence of events that involves glomerular protein leakage as well as tubulo-interstitial inflammation. Excess presence of proteins in the renal tubules leads to tubular uptake of the leaked proteins and hence activates the tubulo-interstitial cells to secrete inflammatory cytokines (64). Persistent inflammation can lead to irreversible damage with extra-cellular matrix deposition and scarring of tissue. The monocyte-macrophage system plays an important role in this process (65). The damage caused by uncontrolled inflammation may in turn aggravate proteinuria (66). This vicious cycle when not broken will lead to rapid progression towards ESRD. Possible interventions in the process are either anti-inflammatory treatment with immunosuppressive drugs or with calcineurin inhibitors. In inflammatory kidney diseases such as IgA nephropathy or primary glomerulonephritis, such treatments can be successful in selected patients (67). Long term treatment however is limited due to side effects such as increased susceptibility to infections. Moreover, calcineurin inhibitors have also direct renal adverse effects like interstitial fibrosis, tubular atrophy and medial arteriolar hyalinosis (68). Therefore, the currently available interventions targeting the inflammatory component of the progressive CKD have not gained wide acceptance, and it might be worthwhile to investigate new anti-inflammatory therapies. In **chapter 5** of this thesis, we therefore investigated the renal effects of oral nicotine in a rat model of spontaneous proteinuria.

Neointima formation and end organ damage

Neointima formation may occur due to the damage to the protective endothelial cell layer lining the blood vessels (69-71). The damaged endothelial cells get activated in response to the injury and initiate an inflammatory response. The injury could be due to shear stress related to high blood pressure, ischemia/reperfusion injury or immunological injuries. Neointima formation is a complex and active process, in which cellular proliferation, cellular death, cell migration and deposition of extra cellular matrix occur simultaneously (10). The neointima consists of vascular smooth muscle cells, extra cellular matrix and inflammatory cells. The formation of neointima leads to decreased luminal patency and reduced blood supply, with subsequent ischemia in the end-organ. Neointima formation in the vessels of transplanted organs is a hallmark of CTD and a major component of long-term organ rejection. As noted above, there is no definitive treatment for CTD. Current standard immunosuppressive therapy with calcineurin inhibitors is successful in preventing or delaying short term rejection, however, CTD is not prevented (72). Moreover, long term administration of CNIs also have vasculotoxic effects (68,73). This indicates the need for additional treatment modalities in renal transplantation to protect against CTD. Since neointima clearly involves an inflammatory component, we studied the effects of oral nicotine on renal neointima formation in two different experimental models of progressive renal failure in **chapter 6** of this thesis.

Finally in **chapter 7** the results of above studies are summarized and discussed. In the same chapter future research perspectives are proposed.

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Chapter 2

Smoking is a Risk Factor for Graft Failure and Mortality after Renal Transplantation

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Chapter 2

Abstract

Smoking in renal transplant recipients (RTR) is an acknowledged cardiovascular risk factor. It is however unclear whether smoking also increases the risk of graft failure (GF). In this study we prospectively assessed the association of current smoking versus past and never smoking with GF and mortality in 604 RTR (age 51.5±12.1 yrs, 55% male). At inclusion 133 (22%) were current smokers, 255 (42%) were past smokers and 216 (36%) never smoked. During follow up of 5.3 [4.7-5.7] yrs, 41 (7%) RTR experienced GF and 95 RTR (16%) died. Current smoking RTR had higher risk of GF compared to never smoking RTR (reference group) (Hazard Ratio (HR) = 3.3 [95%CI] [1.5- 7.1], *P*=0.002). Past smoking RTR had similar risk of GF as never smoking RTR (HR=1.1 (0.5-2.6), *P*=0.7). Current smoking RTR and past smoking RTR were at higher risk for death than never smoking RTR (HR=2.1 [1.1-3.8], *P*=0.016, and HR=2.4 [1.4-4.0], *P*=0.001 respectively).

Smoking after renal transplantation is associated with risk for GF and mortality. Since past smoking is a risk factor for mortality but not for GF smoking cessation may be beneficial for RTR in delaying graft failure in long term.

Introduction

Cigarette smoking is an established risk factor for cardiovascular mortality and cancer (1). A detrimental role of smoking in kidney disease is now also emerging. In the general population, smoking is related to albuminuria and abnormal renal function (2). In prospective studies in patients with diabetes, it has been shown that smoking is a risk factor for development and progression of diabetic nephropathy to end-stage renal disease (3-5). Similar findings have been done in prospective studies in patients primary renal diseases, including lupus nephritis, autosomal polycystic kidney disease and IgA nephropathy, in which smoking was found to be a risk factor for decline of renal function and subsequent progression to end stage renal disease (6-8).

It is, however, less clear whether active smoking is also risk factor for decline of renal function decline and graft failure (GF) after renal transplantation. Prior studies were retrospective in design and investigated pre-transplant cigarette smoking as a risk factor for development of graft failure after transplantation (9-11). To the best of our knowledge there are no prospective studies that have evaluated the effect of post-transplant smoking status on outcome in stable outpatient renal transplant recipients.

We aimed to prospectively investigate whether post-transplant smoking is a risk factor for graft failure or mortality in renal transplant recipient recipients after transplantation.

Patients and Methods

Between August 2001 and July 2003 all outpatients renal transplant recipients (RTR) with a functioning graft for at least one year were asked to participate in a prospective cohort intended to study long-term graft survival. A total of 606 renal transplant recipients signed written informed consent, from an eligible total of 847 (72% consent rate). The patients who consented were comparable to those who did not consent with respect to age, sex, body mass index (BMI), serum creatinine, creatinine clearance, and urinary albumin excretion (12). All participating subjects visited the outpatient clinic at least once a year, and serum creatinine was assessed at every visit. Continuous surveillance system of the outpatient program ensured up-to-date information on patient status. General practitioners or referring nephrologists were contacted in cases of missing data on patient status. Study details have previously been described more extensively (12-14).

Further patient data for this study was obtained from the Groningen Renal Transplant Database. This database holds information on all renal transplantations performed at our center since 1968.

The Institutional Review Board approved the study protocol (METc 2001/039). Funding sources neither had a role in the collection and analysis of data, nor in the publication of the manuscript.

Endpoints

Death-censored graft failure and patient mortality were chosen as primary endpoints. Death-censored graft failure was defined as return to dialysis or re-transplantation. Mortality and graft failure of all RTR was recorded until August 2007. There was no loss to follow-up.

Smoking status

Smoking status was obtained by self-report (questionnaire) at inclusion date. Recipients gave details about their smoking habits, numbers of cigarette smoked and duration of smoking and time of smoking cessation. Smoking status was reported in by 604 of 606 RTR, which were all included in the present analysis. RTR were subdivided into three categories based on the smoking status: never smokers, current smokers and past smokers. Current smokers were those who reported smoking at the time of inclusion (regardless of intensity), past smokers were those who were non-smokers at the time of inclusion but had ever smoked in their life (regardless of duration or time of smoking cessation).

Renal transplant characteristics

Patient characteristics at time of inclusion were obtained from the Groningen Renal Transplant Database. These characteristics were recipients demographics (age, sex, follow-up at time of inclusion, time on dialysis prior to renal transplantation), donor demographics (age, sex, living/ postmortem), body composition (BMI, waist-circumference, body surface area (BSA)), clinical parameters (systolic and diastolic blood pressure, mean arterial pressure (MAP)) immunosuppressive regimen, use of antihypertensive medication, cardiovascular history, laboratory parameters (hemoglobin levels, CRP, albumin, glucose homeostasis, lipids) and renal function (serum creatinine, creatinine clearance, eGFR and albumin excretion rate). All measurements were described previously (12-14).

Statistical analysis

Data were analyzed with SPSS version 14.0 (SPSS Inc. Chicago, IL), and GraphPad Prism version 4.03 (GraphPad Software Inc. San Diego, CA). Normally distributed variables are given as means ± standard deviation, whereas variables with a skewed distribution are given as median [interquartile range]. Differences between the smoking groups were tested for statistical significance with one-way ANOVA for normally distributed variables, with Kruskal-Wallis test for skewed distribution, and with Chi-square test for categorical variables. To assess change in renal function over time, baseline renal function was related to information on renal function obtained at the outpatient clinic until 4 years after baseline. Follow-up date for patients who died with a functioning graft (n = 32) before this time was defined as renal function at the last visit to the outpatient clinic before death, and follow-up for patients with graft failure was defined as renal function at the last visit to the outpatient clinic before starting dialysis. For analyses with graft failure and mortality as outcome, survival analyses were performed according to Kaplan-Meier and compared by overall log-rank tests. Further survival analyses, in which was adjusted for potential confounders was performed using Cox proportional hazard regression. Since smoking is an exposure it is inappropriate to adjust for variables that may be in the causal pathway between smoking and outcomes (15). To avoid over-adjustment by adjustment for variables that are intermediates between smoking and outcomes (15), we made a careful selection of variables that are unlikely to be intermediates between smoking and outcomes, but could confound the association by being associated with both smoking and outcomes. These variables are age, sex and time since transplantation. Although creatinine clearance and proteinuria may be in the causal pathway, we also considered it important to adjust for these variables, particularly for graft failure as end-point, because baseline values may importantly determine outcome. Proportionality assumptions were tested using Schoenfeld tests and log-minus-log survival plots. A two-sided P-value less than $P<0.05$ indicated statistical significance.

Results

Smoking data were available in 604 RTR with a mean (SD) age of 51.5 (12.1) years. Time of inclusion was at a median [interquartile range] of 6.0 [2.6-11.5] years post-transplantation. At the time of inclusion, 216 (36%) RTR had never smoked, 255 (42%) smoked in the past, and 133 (22%) were current smokers. Baseline characteristics according to smoking status are shown in table 1. Past smokers were significantly older than current smokers and never smokers. Past smokers were also more frequently men than never smokers, with current smokers in between. Similar trends and significant differences were present for history of myocardial infarction, waist circumference and statin use, while C-reactive protein was lowest in past smokers. Prednisolone dose and serum creatinine were highest in current smokers.

Table 1 Baseline characteristics subdivided according to the smoking status.

	Smoking status			
	Non Smoker	Past Smoker	Current Smoker	**p-value**
N (%)	216 (36)	255 (42)	133 (22)	
Recipient demographics				
Age, yrs	50±14	53±11	50±12	0.007
Sex- Men, n (%)	102 (47)	156 (61)	73 (55)	0.01
Time since transplantation, yrs	7.0 [3.5-11.7]	5.6 [2.5-10.8]	5.6 [2.2-11.9	0.1
Dialysis time, months	27 [14-52]	28 [12-49]	25 [14-43]	0.5
Duration of follow-up, yrs	5.3 [4.7-5.7]	5.2 [4.5-5.7]	5.2 [4.2-5.8]	0.29
CVD history				
Myocardial infarction, n (%)	11 (5)	27 (11)	10 (8)	0.08
TIA / CVA, n (%)	9 (4)	17 (7)	7 (5)	0.5
Body composition				
Body mass index, kg/m^2	26±5	26±4	26±4	0.3
Waist circumference, cm	96±14	99±14	96±13	0.1
Waist circumference - Men, cm	96±12	102±12	99±13	0.001
Waist circumference - Women, cm	97±15	93±14	92±12	0.06
Blood pressure				
Systolic pressure, mmHg	151±21	154±23	154±25	0.5
Diastolic pressure, mmHg	90±10	90±10	90±10	0.7
Mean arterial pressure mmHg	140±15	141±16	141±16	0.5
Anti-hypertensive drugs, n (%)	184 (85)	226 (89)	118 (89)	0.5
ACE or AT II inhibitors, n (%)	67 (31)	95 (37)	39 (29)	0.2
Anti hypertensive drugs	2 [1-3]	2 [1-3]	2 [1-3]	0.08
Glucose homeostasis				
Fasting insulin, pmol/l	11.9 [8.5-17.0]	11.2 [8.3-15.4]	9.6 [7.4-15.8]	0.06
Glucose, mmol/l	4.6 [4.1-5.0]	4.5 [4.1-5.0]	4.6 [4.2-5.0]	0.6
HOMA	2.4 [1.6-3.9]	2.3 [1.6-3.3]	2.0 [1.5-3.4]	0.1

Diabetes, n (%)	45 (21)	46 (18)	16 (12)	0.1
Lipids				
Total cholesterol, mmol/l	6±1	6±1	6±1	0.3
LDL cholesterol, mmol/l	4±1	4±1	4±1	0.8
Statin use, n (%)	100 (46)	140 (55)	60 (45)	0.09
CRP, mg/l	2.0 [0.8-4.2]	1.9 [0.7-4.0]	3.4 [1.0-7.8]	0.01
Albumin, g/dl	4±3	4±4	4±3	0.9
Hemoglobin, mmol/l	8.5 [7.9-9.1]	8.6 [8.1-9.2]	8.7 [8.1-9.5]	0.1
Anti rejection medications				
Immunosuppressant				
Calcineurin inhibitor, n (%)	172 (80)	203 (80)	99 (74)	0.4
Prednisolone dose, (mg/day)	10 [7.5-10]	10 [7.5-10]	10 [10-10]	0.01
Proliferation inhibitor				
Azathioprine, n (%)	82 (38)	71 (28)	44 (33)	0.9
Mycophenolate mefotil, n (%)	76 (35)	113 (44)	60 (45)	
Donor demographics				
Age, yrs	36±15	38±15	37±16	0.6
Sex - Men, n (%)	115 (54)	139 (55)	72 (55)	0.9
Living kidney donor, n (%)	22 (10)	42 (17)	19 (14)	0.14
Postmortem donor, n(%)	194 (90)	213 (84)	114 (86)	
Renal function				
CrCl at inclusion, ml/min	66 [53-79]	64 [53-78]	64 [47-76]	0.4
Serum creatinine, (µmol/L)	132 [110-159]	133 [111-168]	143 [117-177]	0.06
Proteinuria ≥0.5 g/24hr, n (%)	54 (25)	68 (27)	46 (35)	0.1

Normally distributed variables are presented as mean ± SD and differences were tested with one way analysis of variance. Variables with a skewed distribution as presented as median (25-75 percentiles) and differences were tested with Kruskal-Wallis test. Categorical variables are presented as number (column percentage) and differences were tested with chi-square test.

Abbreviations: 1. TIA: Transient ischemic attack, 2. CVA: Cereberovascular accident, 3. ACE: Angiotensin converting enzyme 4. ATII: Angiotensin II receptor, 5. HOMA: Homeostasis model assessment, 6. LDL: Low density lipoprotein, 7. CRP: C-reactive protein, 8. CrCl: Creatinine clearance, 9. GFR: Glomerular filtration rate.

During the first 4 years of follow-up, decline in renal function was faster in current smokers than in past or never smokers (-4.2 (0.54) ml/min/yr vs. -1.9 (0.46) ml/min/yr and -1.5 (0.53) ml/min/yr resp., p=0.003, figure 1).

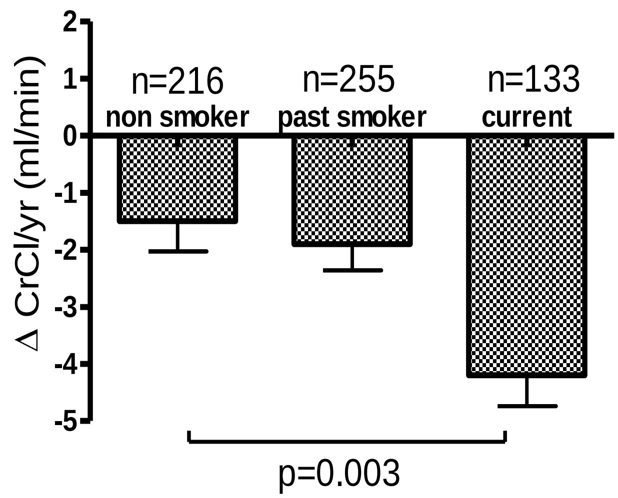

Figure 1 Decline in kidney function as measured by creatinine clearance in non-smoker, past-smoker and current smoker.

During follow-up for 5.3 [4.7-5.7] years, incidence of graft failure was higher in the group of current smokers than in the groups of past and non-smokers (19 (14%) vs. 13 (5%) and 10 (4%) resp., figure 2A, p<0.001). Mortality was higher both in the groups of current and past smokers than in the group of non-smokers (24 (18%) and 52 (20%) vs. 19 (9%), figure 2B, p<0.005).

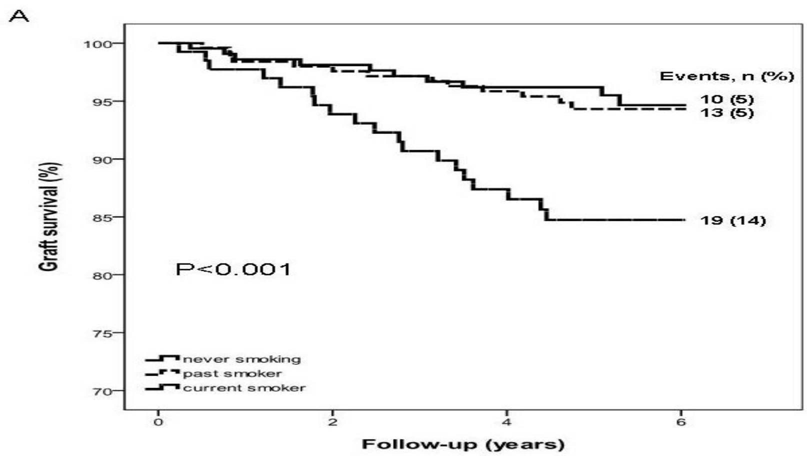

Figure 2A Kaplan Meier analyses for death censored graft survival in renal transplant recipients subdivided into current, past and never smoking.

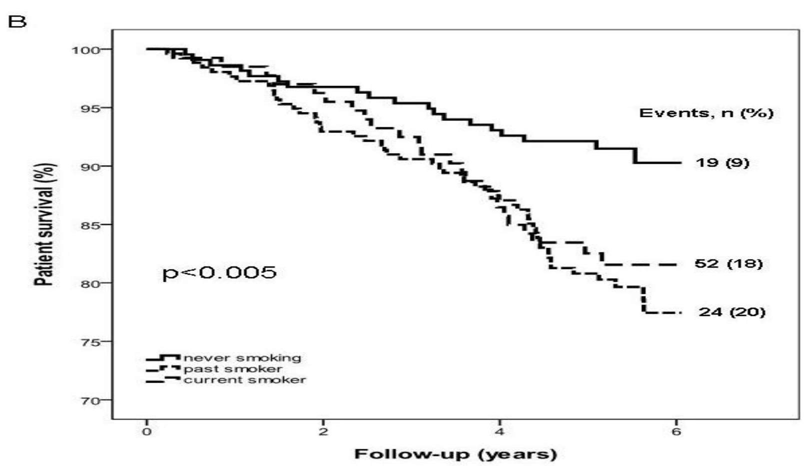

Figure 2B Kaplan Meier analyses for mortality renal transplant recipients subdivided into current, past and never smoking

In further cox-regression analyses, the associations of current smoking with graft failure and of current and past smoking with mortality remained independent of age, sex, baseline creatinine clearance and baseline proteinuria (table 2).

Table 2

	Never	Past		Current	
	HR (reference)	HR [95% CI]	*P*	HR [95% CI]	*P*
Graft failure					
Model 1	1.0	1.1 [0.5-2.6]	0.7	3.3 [1.5-7.1]	0.002
Model 2	1.0	1.2 [0.5-2.7]	0.7	3.2 [1.5-7.0]	0.003
Model 3	1.0	1.6 [0.7-4.1]	0.3	3.3 [1.5-7.2]	0.003
Model 4	1.0	1.6 [0.6-3.8]	0.3	2.4 [1.1-5.6]	0.03
Mortality					
Model 1	1.0	2.4 [1.4-4.0]	0.001	2.1 [1.1-3.8]	0.02
Model 2	1.0	2.4 [1.4-4.0]	0.002	2.5 [1.3-4.5]	0.004
Model 3	1.0	2.2 [1.3-3.8]	0.004	2.1 [1.1-3.8]	0.02
Model 4	1.0	2.2 [1.3-3.8]	0.004	2.0 [1.1-3.7]	0.03

Table 2. Univariate and multivariate analyses of past and current smoking as risk factors for graft failure and mortality.

Model 1: Crude
Model 2: Model 1 + recipient age, gender and time since transplantation.
Model 3: Model 2 + creatinine clearance.
Model 4: Model 3 + proteinuria.

Discussion

In this study, we found that active smoking is associated with a relatively fast decline of renal function after transplantation, translating into it being an independent risk factor for development of graft failure after renal transplantation. We also found that current smoking and past smoking both are independent risk factors for mortality after transplantation.

We also found that 22% of our large, stable outpatient RTR population were current smokers and 42% were past-smokers. Data on smoking habits post-transplantation are scarce. In the ALERT trial, with baseline measurements in 1996-1997, prevalence of current smokers among outpatient RTR beyond 6 months after transplantation was reported to be 18.9% (16). No data on past smoking were presented. In a more recent cross-sectional study among RTR in Germany, 12.5% were current smokers and 46.5% were past smokers (17). So, the prevalence of current smokers in our study was relatively high and the percentage of past smokers was relatively low compared to other studies. Possibly, stimulation of quitting smoking after transplantation was less well-established in our clinic than in the other clinics at the time baseline measurements were performed.

To the best of our knowledge our study is the first to prospectively assess potential association of post-transplant assessment of smoking status with development of graft failure and mortality after transplantation. This is important, because it has been estimated that shortly after transplantation approximately 30% of patients that smoke while being pre-transplant, quit smoking after transplantation (17) . Prior studies were retrospective in design or investigated pre-transplant cigarette smoking as a risk factor for development of graft failure and mortality after transplantation (9-11). In a substudy of the ALERT trial, the only other study to the best of our knowledge to date that assessed the association between post-transplant smoking status and outcome, it was found that current smoking as compared to current non-smoking was a risk factor for graft failure without inclusion of death in the end-point, with a hazard ratio of 2.29, which dropped to 1.77 when death was added to the combined end-point (18). This implies that current smoking as compared to current non-smoking was a less strong risk factor for death than for graft failure in this trial (18). If we take our data into account, it seems very important to make the distinction between past-smoking and never smoking among current non-smokers, because never smokers have the lowest risk, while in the ALERT trial past-smokers were in the control group, which may have given an underestimation of the actual risk held by current smoking. The mechanism underlying the association between current smoking and graft failure cannot be established in our data or that of the ALERT trial. Interestingly, however, we found that past smoking is a risk factor for mortality but not for graft failure, which suggests that atherosclerosis of the native vasculature plays no – or a minor – role in the association between smoking and graft failure in RTR. A role for the intra-renal vasculature has been suggested in a cross-sectional study of 279 renal transplant biopsies, where it was found that severity of vascular intimal fibrous thickening was associated with current smoking, whereas the degree of arteriolar hyalinosis and chronic sclerosing nephropathy were associated with time since transplantation (19). Thus, a vascular effect of current smoking seems to prevail, even in transplanted kidneys. In line with this is the finding that post transplant smoking has been found to a risk factor of coronary artery disease (CAD) in heart transplant patients (20;21).

Strengths of our study are its prospective design, its collection of data not only on current smoking, but also on past and never smoking and the large size of the population. There are also several limitations to the study. First, all the data on smoking status are self reported and based on a questionnaire that was applied once at the baseline of the study, so we have no data on smoking status of recipients and donors before transplantation or on change in smoking status beyond baseline of the study. However, any un-assessed change in smoking status beyond the baseline of our study may only result in weakening of otherwise stronger associations. So, the associations that we found are at worst an underestimation of truly existing associations. Second, the relatively late (median 6 years post-transplant) time of inclusion may have imparted a survival bias on those included in the study, as smokers may already lost their graft or died before the time of the study recruitment. However, inclusion of patients as they are appearing at an outpatient clinic gives a picture of actual clinical, which is may less well be the case when subjects are included at a certain time point after transplantation. Third, our study was too small to allow for meaningful analyses of dose-effect relationships of intensity of smoking or degree of total exposure. It would be interesting if future studies in larger databases or combinations of databases could address this issue.

In conclusion, current smoking poses RTR at increased risk for graft failure and mortality. Past smoking is a risk factor for mortality but not for graft failure, suggesting atherosclerosis of the native vasculature to play no important role in the association between smoking and graft failure in RTR. Stop smoking could particularly benefit RTR in terms of preservation of renal function.

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Chapter 3

Former Smoking is a Risk Factor for Chronic Kidney Disease after Lung Transplantation

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Chapter 3

Abstract

Chronic kidney disease (CKD) is a common complication after lung transplantation (LTx). Smoking is a risk factor for many diseases, including chronic kidney disease (CKD). Smoking cessation for >6 months is required for LTx-enlistment. However, the impact of smoking history on CKD development after LTx remains unclear. We investigated the effect of former smoking on CKD and mortality after LTx. CKD was based on glomerular filtration rate (GFR) (^{125}I-iothalamate-measurements). GFR was measured before and repeatedly after LTx. 134 patients never smoked and 192 previously smoked for a median of 17.5 pack years. At 5 yrs after LTx, overall cumulative incidences of CKD-III, CKD-IV and death were 68.5, 16.3 and 34.6%, respectively. Compared to never smokers, former smokers had a higher risk for CKD stages III (HR [95%CI]=1.69 [1.27-2.24]) and IV (HR=1.90 [1.11-3.27]), but not for mortality (HR=0.99[0.71-1.38]). Adjustment for potential confounders did not change results. Thus, despite cessation, smoking history remained a risk factor for CKD in LTx recipients. Considering the increasing acceptance for LTx of older recipients with lower baseline renal function and an extensive smoking history, our data suggest that the problem of post-LTx CKD may increase in the future.

Introduction

Chronic kidney disease (CKD) is a serious complication after lung transplantation (LTx). In most LTx-recipients renal function deteriorates progressively, often resulting in CKD (1). Progression from CKD to end-stage renal disease (ESRD) currently develops in between 3-10% of LTx recipients (2-4). Recipient age and life-expectancy after LTx are increasing, so it is likely that the a priori risk for CKD after LTx is increasing concomitantly, with possible consequences for the number of LTx recipients that develop ESRD on the long term (5).

Approximately 60% of LTx recipients have a history of smoking (6). This is the highest rate reported among recipients of solid organs: former smokers account for 51-54% of renal-transplant recipients (7,8), 42-50% of liver-transplant recipients (9), and 45% of heart-transplant recipients (5). Moreover, many former smokers amongst LTx recipients smoked heavily, as around 40-45% of them undergo LTx because of end-stage pulmonary emphysema, which is largely attributable to heavy smoking (6). Pulmonary emphysema, cardiovascular disease and lung cancer are well-known complications of smoking (10-12). It may be less well-known that smoking is also a risk factor for CKD (13,14). We wondered whether, even after smoking-cessation, smoking history could be relevant for morbidity after LTx, in particular CKD, because of the high prevalence of both CKD and former smoking in LTx recipients.

Transplant centers, including our own, commonly require that patients have stopped smoking for at least six months before being enlisted for LTx (15,16). In the current study, we analyzed the potential association between smoking history before LTx and development of CKD after LTx in a large single center cohort of LTx recipients. In addition, we analyzed the impact of past smoking on mortality and causes of death after LTx.

Patients and methods

A total of 370 lung transplantations, of which 22 heart-lung transplantations were performed at the University Medical Center of Groningen (UMCG), the Netherlands between 1990 and 2008. Pediatric transplantations (n=17) and re-transplantations (n=13) were excluded. Quantitative data on smoking history were obtained from patient records. We excluded , 14 patients of the remaining 340 patients from further analyses because of lack of data on smoking history, leaving a total of 326 patients eligible for evaluation. Further clinical information of all individuals was gathered at baseline and during follow-up. Consent for the use of patient data was obtained from all patients prior to transplantation.

Smoking history

Smoking cessation for at least 6 months is a requirement for LTx enlistment. Returning to active smoking after LTx is very strongly disapproved and occurs, to the best of our knowledge, only exceptionally. Smoking history prior to transplantation was quantified by number of pack years. One pack year is defined as 20 cigarettes (one pack) smoked per day for one year. Patients were categorized into never and former smokers and the latter were further categorized according to number of pack years: 1-10, 11-25 and >25 pack years.

GFR measurements

Glomerular filtration rate (GFR) measurements were performed by constant infusion of radiolabelled tracers ^{125}I-Iothalamate and ^{131}I-Hippuran as described before (17-19). This method is considered a gold standard method for GFR measurement, has a day-to-day coefficient of variation of 2.5 % (19) and is used for the development and validation of novel renal function equations (20). Height and weight were measured in every patient before every GFR measurement. Body surface area (BSA) was calculated as

$0.007184*\text{weight}^{0.425}*\text{length}^{0.725}$ (21), and GFR was expressed per 1.73 m^2 of BSA. GFR measurements were performed routinely at out-patient basis, before transplantation and at regular time-intervals after transplantation.

Endpoints of the study

Primary endpoints of the study were CKD-III and CKD-IV. CKD-III and CKD-IV were defined as GFR<60 ml/min/1.73m^2, and GFR<30 ml/min/1.73m^2 respectively, according to the cut-off values in the NKF K/DOQI guidelines (22).

We used GFR measurements by radiolabelled Iothalamate for the definition of CKD because in LTx recipients creatinine-based GFR estimations are biased by the large variation in muscle mass (23). For the primary analyses, definition of CKD was based on the first GFR measurement below the cut-off values defining the stages of CKD. We did this because beyond 2 years after LTx we measured GFR with 2-year intervals. Consequently, a proportion of patients with one measurement below the CKD-threshold lacked follow-up until a confirmatory measurement. Defining CKD based on at least 2 measurements below the CKD-threshold would result in underestimation of true cumulative incidence of CKD. Moreover, in patients that had a GFR measurement below the cut-off, only 2.4% the second GFR measurement was inconsistent with the former measurement. This assured us that use of this definition for endpoints was appropriate. However, to consent with the NKF K/DOQI guidelines CKD should be confirmed by a second measurement >90 days later, we performed secondary analyses with the endpoints defined as such. We furthermore performed a secondary analysis with doubling of serum creatinine (DSC) as alternative renal endpoint to check consistency across renal endpoints. DSC was defined as a doubling of serum creatinine, confirmed by a similar value >90 days after the initial doubling.

ESRD was defined as the requirement of renal replacement therapy. We recorded occurrence of ESRD and death until January 2010. Causes of death were coded according to the international Classification of Diseases (ICD-9).

Immunosuppressive Regimens

Immunosuppressive protocols used over time are described in more detail elsewhere (24). In short, maintenance immunosuppression consists of a calcineurin inhibitor (CNI) based immunosuppressive regimen, combined with steroids and azathioprine (Immuran; GlaxoSmithKline,United Kingdom). Cyclosporine (Neoral; Novartis,Switzerland) was the CNI used until 2001. The targeted whole blood trough level of CsA was initially 400 µg/L, tapering to 150 µg/L within the first three weeks. Tacrolimus (Prograft; Astellas,The Netherlands) was the CNI used from 2001 onwards. Between 2001-2004 target trough levels were 20 µg/L during the first three weeks, 15 µg/L until the third month and 10-12 µg/l thereafter. Lower peri-operative tacrolimus trough levels were targeted from 2004: 15-18 µg/L during the first three weeks, 12-15 µg/L until the third month, and 10-12 µg/L thereafter. We further decreased target levels to 8-10 µg/L in the case of EBV-reactivation (24). Prednisolone dose was 0.2mg/kg/d until the third month and 0.1 mg/kg/d thereafter. Azathioprine dose was 1.5-3 mg/kg/d. Induction consisted of ATG (Thymoglobulin; Pasteur-Merieux,France) until 2001 and Basiliximab induction thereafter (Simulect; Novartis,Switzerland). We switched some patients from Azathioprine to Mycophenolate Mofetil (Cellcept; Roche,Switzerland). Patients received P.Jeroveci prophylaxis, Herpes prophylaxis and CMV-prophylaxis (at-risk patients only, from 2001 onwards).

Statistical analysis

Data were analyzed with SPSS version 18.0, STATA version 11.2 and R **version 2.12.0.** The data were obtained from preexistent databases and from patient chart review. Categorical variables are reported as frequencies and percentages. Variables with normal distribution are presented as mean with standard deviation (SD) and variables with a skewed distribution are presented as median with interquartile range [IQR].

Recipient characteristics are shown according to categories of smoking habits prior to transplantation. We divided patients into never smokers and former smokers and subdivided the latter category into three categories according to number of pack years. Differences between groups were tested by one-way ANOVA for linear effects for continuous variables and Chi Squared-test for categorical variables. We performed adjustment for multiple comparisons over time using Bonferroni correction.

CKD-III, CKD-IV and all-cause mortality were endpoints in this study. Cumulative incidences of CKD-III and CKD-IV were calculated for never smokers and groups of former smokers using competing risk analysis. Differences between smoking exposure groups were tested with Gray's test for competing risks data (25,26). We calculated population attributable risk as (proportion exposed*((adjusted hazard ratio-1)/adjusted hazard ratio)*100% (27). The association of former smoking with CKD-III and IV was modeled with Cox cause-specific hazards regression (28) and the association with mortality with standard Cox proportional hazards regression. Variables that we selected as covariates in multivariate analyses were patient demographics, variables that were different between the smoking exposure groups at baseline and variables previously found to be risk factors with renal risk after LTx (29,30). Linearity of continuous covariates was assessed using multivariable fractional polynomials in R. According to this package optimal model-fit for GFR would be obtained by a 1000/GFR transformation, whereas age, BMI and pack years were either best fitted in a linear non-transformed way or did not contribute to the model. We therefore used a 1000/GFR-transformation of GFR in the analyses. To assess dose-dependency, the number of pack years was analyzed as continuous variable. We performed secondary analyses without the never smokers and stratified for age (< 47 and ≥ 47 years, population median) and for diagnosis (COPD vs. non-COPD) to rule out that (dose) effects were solely based on the differences in characteristics of never smokers and former smokers. Moreover, in further secondary analyses, we repeated the multivariate analyses with CKD-III and CKD-IV confirmed by a second measurement >90 days later and with doubling of serum creatinine, also confirmed by a second measurement >90 days later as alternative renal endpoints. Lastly, as body dimensions may change substantially after LTx we performed a secondary analysis with body-surface-area as time-dependent variable. Possible interactions between variables were also assessed. Hazard ratios (HR) are reported with 95% confidence interval [95%CI]. Final results were considered significant at a level <0.05.

Results

Quantitative data on previous history of smoking were available in 326 (96%) patients: 134 (41.1%) patients had never smoked and 192 (58.9%) patients had smoked a median [IQR] of 17.5 [10-30] pack years. Baseline characteristics of never smokers and former smokers stratified for number of pack years are shown in table 1.

	Never smokers	Former smokers			
	0 Pack years	1-10 Pack years	11-25 Pack years	>25 Pack years	*P-value*
Number of patients	134	62	74	56	
Pack years	0 ± 0	6.5 ± 3.0	18.1 ± 4.7	38.7 ± 12.1	
Male Sex (n, %)	73 (54.3)	26 (41.9)	40 (54.1)	27 (48.2)	*0.37*
Recipient age* (years)	36.8 ± 12.5	48.5 ± 8.2	50.7 ± 6.9	55.1 ± 5.6	*<0.001*
Race					*0.88*
Whites	131 (99.3)	62 (100)	73 (98.6)	56 (100)	
Blacks	3 (0.7)	0 (0)	1 (1.4)	0 (0)	
Glomerular filtration rate (ml/min/1.73m2)	108 ± 26	98 ± 18	101 ± 15	93 ± 17	*0.04*
Pre-transplantation SCr (µmol/L)	77 ± 19	80 ± 18	79 ± 15	79 ± 16	*0.68*
Body mass index (BMI) (kg/m²)	21.0 ± 3.5	22.1 ± 3.6	23.3 ± 4.0	23.2 ± 3.2	*<0.001*
Hypertension (n, %)	26 (19.5)	15 (24.2)	13 (17.6)	8 (14.3)	*0.59*

Diabetes Mellitus (n, %)	23 (17.3)	2 (3.2)	4 (5.4)	2 (3.6)	*0.002*
Pulmonary diagnosis (n, %)					*<0.001*
COPD	4 (3.0)	10 (16.1)	25 (33.8)	39 (69.6)	
α1-antitrypsin-deficiency (*AAT)*	2 (1.5)	23 (37.1)	31 (41.9)	10 (17.9)	
Cystic Fibrosis (CF)	61 (45.5)	3 (4.8)	1 (1.4)	0 (0)	
Pulmonary Hypertension (PH)	20 (14.9)	9 (14.6)	4 (5.4)	3 (5.4)	
Pulmonary Fibrosis (PF)	28 (20.9)	12 (19.4)	10 (13.5)	4 (7.1)	
Other	19 (14.1)	5 (8.1)	3 (4.1)	0 (0)	
Type of transplantation (n, %)					*<0.001*
Unilateral	21 (15.7)	14 (22.6)	15 (20.3)	19 (33.9)	
Bilateral	100 (74.6)	46 (74.2)	57 (77.0)	36 (64.3)	
Combined organ	13 (9.7)	2 (3.2)	2 (2.7)	1 (1.8)	
Immunosuppressive regimen (n, %)					*0.045*
CsA-based	57 (42.5)	35 (56.5)	39 (52.7)	16 (28.6)	
Tac-based	77 (57.5)	27 (43.5)	35 (47.3)	40 (71.4)	
Transplant series (n, %)					*0.043*
1990-1996	23 (17.2)	10 (16.1)	10 (13.5)	5 (8.9)	
1997-2000	34 (25.4)	24 (38.7)	24 (32.4)	11 (19.6)	
2001-2004	37 (27.6)	16 (25.8)	15 (20.3)	12 (21.4)	
2005-2008	40 (29.9)	12 (29.7)	22 (29.7)	28 (50)	

* Recipient age at time of transplantation. sCr: serum creatinine, COPD: Chronic obstructive pulmonary disease, CsA: cyclosporin, Tac: Tacrolimus. Differences between groups were tested by one-way ANOVA for linear effects.

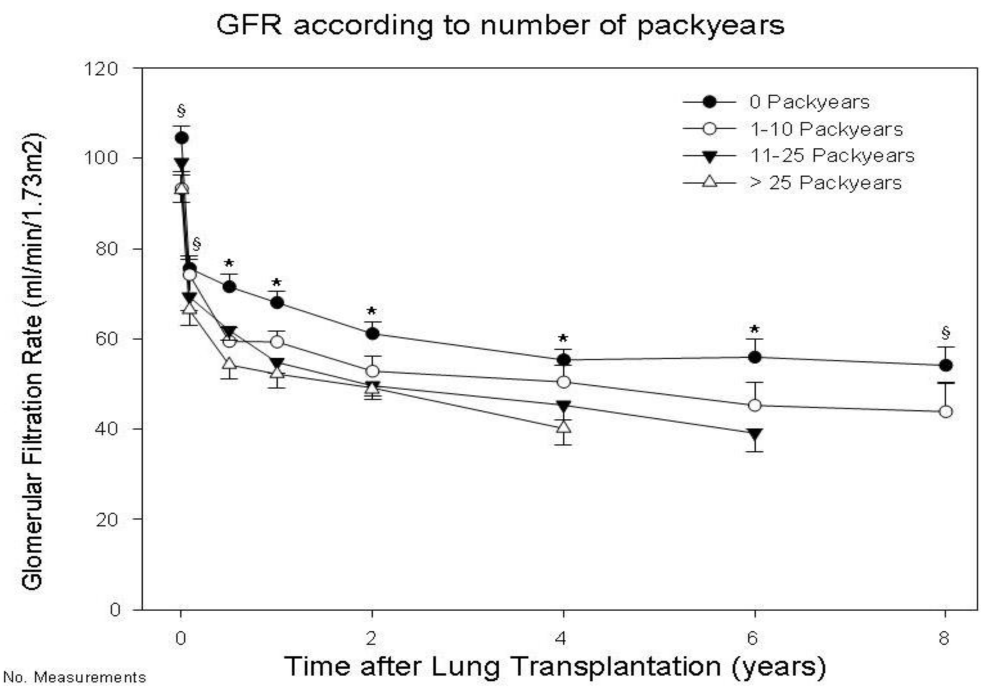
Overall, the former smokers were older, had higher BMI, more often had pulmonary diagnoses chronic obstructive pulmonary disease (COPD) or α1-antitrypsine-deficiency, (as opposed to cystic fibrosis, pulmonary fibrosis or pulmonary hypertension in never smokers), more often underwent unilateral lung transplantation and had lower GFR before transplantation. Comparing subgroups of former smokers the patients with higher numbers of pack years were found to be older, were more often transplanted for COPD and more often received Tacrolimus-based maintenance immunosuppression. A relatively large proportion of heavy former smokers was transplanted in the most recent stratum. This reflects recent changes in acceptance policy for LTx enlistment.

Other characteristics were similar for the smoking exposure groups. Cyclosporine and Tacrolimus trough levels were similar for never smokers and all groups of former smokers in all time-intervals during follow-up after transplantation (table 2).

Cyclosporin (ng/mL)		**0-1 month**	***N***	**1-6 months**	***N***	**6-24 months**	***N***	**>24 months**	***N***
Never smokers	0 Pack years	293 ± 53	*56*	195 ± 56	*53*	177 ± 41	*45*	182 ± 28	*39*
Former smokers	1-10 Pack years	299 ± 40	*34*	175 ± 25	*32*	166 ± 30	*27*	170 ± 28	*25*
	11-25 Pack years	294 ± 43	*37*	180 ± 25	*31*	175 ± 34	*28*	165 ± 45	*25*
	>25 Pack years	280 ± 35	*16*	180 ± 28	*16*	172 ± 22	*15*	172 ±17	*10*
Tacrolimus (ng/mL)									
Never smokers	0 Pack years	14.9 ± 2.8	*71*	10.9 ± 2.3	*55*	10.2 ± 1.7	*55*	9.8 ± 1.3	*40*
Former smokers	1-10 Pack years	14.8 ± 2.0	*26*	10.8 ± 1.6	*22*	10.7 ± 2.0	*21*	10.1 ± 1.4	*14*
	11-25 Pack years	15.2 ± 2.1	*33*	11.4 ± 1.9	*32*	10.7 ± 2.3	*31*	10.0 ± 1.8	*18*
	>25 Pack years	14.3 ± 1.9	*35*	10.7 ± 1.7	*29*	10.3 ± 1.5	*28*	9.8 ± 1.2	*20*

P > 0.05 for Cyclosporin and Tacrolimus trough levels in never smokers and groups of former smokers at all-time intervals after lung transplantation. Differences between groups were tested by one-way ANOVA for linear effects.

Mean baseline GFR was 102±22 ml/min/1.73m^2. Baseline GFR was higher in never smokers than in former smokers, but this difference did not persist after adjustment of GFR for recipient age. Renal function declined considerably after LTx, on average -43±24% within 2 years after LTx. The decline was particularly steep in the first 6 months after LTx, slowly leveling off beyond 12 months after LTx (figure 1). The slope of GFR decline in the first year after LTx was steeper in former smokers than in never smokers (-34±20 and -25±18% resp., P=0.005). Former smokers generally had a significantly lower mean GFR than never smokers from 6 months after LTx onwards. The former smokers with a history of >25 pack years of smoking had lowest mean GFR at all-time points.

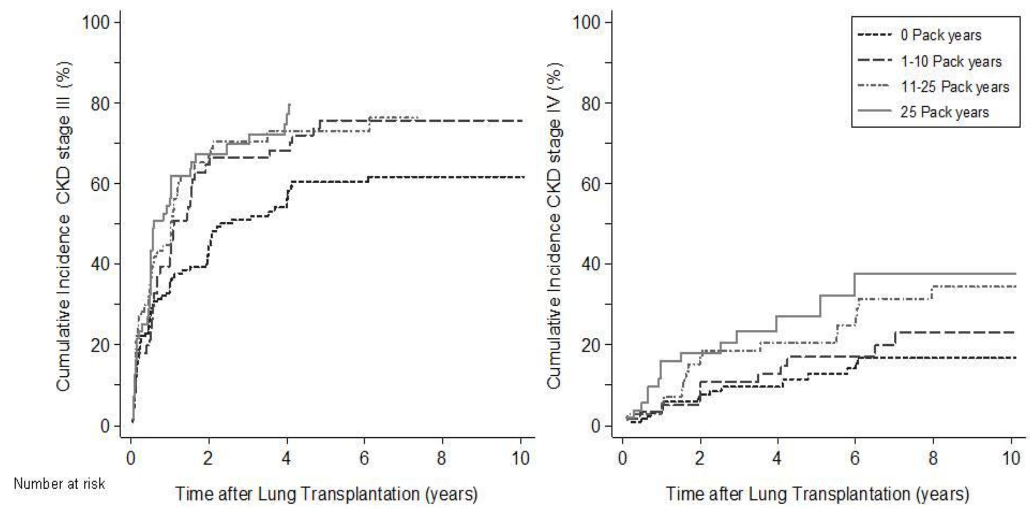


No. Measurements							
0 Packyears	118	83	83	68	50	28	19
1-10 Packyears	56	47	47	32	27	16	10
11-25 Packyears	67	54	58	39	28	20	<10#
>25 Packyears	51	41	39	38	30	<10#	<10#

Means with standard error of GFR relative to body-surface-area per group according to groups based on number of pack years (0 Pack years, 1-10 Pack years, 11-25 Pack years, >25 Pack years). Differences between groups were tested by one-way ANOVA for linear effects. §P<0.05, NS after Bonferroni correction, *P<0.006, significant after Bonferroni correction # Mean estimates not reported for <10 patients.

The overall cumulative incidence of CKD-III at 5 years after LTx, taking competing risk of death into account, was 68.5%, with a cumulative incidence of 58.2% in never smokers vs. 74.4% in former smokers ($P<0.001$). This cumulative incidence was 72.6%, 74.7% and 75.8% ($P<0.001$) in former smokers with a history of 1-10, 11-25 and >25 pack years of smoking, respectively. The overall cumulative incidence of CKD-IV at 5 years after LTx, taking competing risk of death into account, was 16.3% with a cumulative incidence of 10.7% in never smokers vs. 20.2% in former smokers ($P=0.004$). This cumulative incidence of CKD-IV at 5 years after LTx was 15.0%, 20.4% and 26.9% ($P=0.004$) in former smokers with a history of 1-10, 11-25 and >25 pack years of smoking, respectively.
Fifteen (5% of total) patients (mean age at LTx 38±13 years) progressed to ESRD at a mean follow-up of 7.2±4.1 years. Of those patients, 7 had COPD and 7 had cystic fibrosis. Current average follow-up of patients alive is 5.3±4.2 years; hence the proportion of ESRD might increase with longer follow-up. We refrained from further (multivariate) analyses on determinants of ESRD, because of the small number of patients with ESRD.

In univariate Cox proportional Hazard analyses, former smokers had an increased risk of CKD-III (HR [95%Confidence Interval(CI)]=1.69 [1.27–2.24], $P=0.001$) and CKD-IV (HR=1.90 1.11-3.27], $P=0.02$) compared to never smokers. Among the former smokers, there was an increase in risk of CKD-III per group according to number of pack years, with HRs of 1.57 [1.09-2.25], $P=0.015$, 1.65 [1.15-2.36], $P=0.006$ and 1.92 [1.32-2.79], $P=0.001$ for patients with a history of 1-10, 11-25 and >25 pack years of smoking respectively, compared to never smokers. The same was true for CKD-IV, with HRs of 1.30 [0.63-2.70], $P=0.48$, 1.96 [1.04-3.71], $P=0.04$ and 2.78 [1.41-5.46], $P=0.003$ for the respective categories of former smokers compared to never smokers (Figure 2).



Number at risk

0 Pack years	134	6	22	7	6	134	76	54	37	22
1-10 Pack years	60	14	11	2	2	62	41	30	19	12
11-25 Pack years	73	11	1	0	0	74	40	28	24	13
>25 Pack years	56	6	0	0	0	56	28	14	9	4

Cumulative incidences of CKD-III (left, (P<0.001)) and CKD-IV (right, (P<0.001)) according to groups based on number of pack years (0 Pack years, 1-10 Pack years, 11-25 Pack years, >25 Pack years). Differences between groups were tested with grays test for competing risks data.

	CKD stage III						CKD stage IV					
Characteristic	**Univariate**			**Multivariate**			**Univariate**			**Multivariate**		
	HR	*[95% CI]*	*P-value*	*HR*	*[95% CI]*	*P-value*	*HR*	*[95% CI]*	*P-value*	*HR*	*[95% CI]*	*P-value*
Pack years	1.19	[1.09-1.28]	<0.001	1.25	[1.10-1.42]	<0.001	1.40	[1.20-1.62]	<0.001	1.46	[1.17-1.83]	<0.001
Male sex	1.00	(reference)	-	1.00	(reference)	-	1.00	(reference)	-	1.00	(reference)	-
Female sex	0.87	[0.77-0.99]	0.04	0.88	[0.75-1.03]	0.12	1.11	[0.87-1.41]	0.41	1.06	[0.80-1.40]	0.69
Age - years	1.00	[0.99-1.00]	0.97	1.02	[1.00-1.05]	0.03	1.04	[1.01-1.06]	0.005	1.02	[0.98-1.06]	0.47
GFR * - ml/min/1.73m2	1.04	[1.00-1.08]	0.01	1.05	[1.00-1.11]	0.04	1.07	[1.00-1.15]	0.05	1.15	[1.03-1.28]	0.01
BMI - kg/m2	1.03	[0.99-1.07]	0.07	0.97	[0.92-1.02]	0.19	1.05	[0.99-1.11]	0.13	1.01	[0.93-1.09]	0.88
Hypertension	1.16	[0.83-1.61]	0.38	1.03	[0.65-1.63]	0.89	1.35	[0.74-2.44]	0.33	1.37	[0.58-3.27]	0.64
Diabetes Mellitus	0.64	[0.39-1.04]	0.07	0.69	[0.38-1.25]]	0.22	0.58	[0.21-1.58]	0.28	0.64	[0.19-2.20]	0.48
Pulmonary diagnosis												
COPD	1.00	(reference)	-	1.00	(reference)	-	1.00	(reference)	-	1.00	(reference)	-
α1-antitrypsin-deficiency	0.69	[0.47-1.01]	0.06	0.97	[0.62-1.53]	0.90	0.57	[0.30-1.08]	0.08	0.74	[0.35-1.57]	0.44
Cystic Fibrosis	0.44	[0.29-0.68]	0.001	1.31	[0.63-2.73]	0.47	0.34	[0.15-0.75]	0.008	1.48	[0.41-5.44]	0.55
Pulmonary Hypertension	0.73	[0.46-1.16]	0.18	1.24	[0.62-2.47]	0.54	0.42	[0.16-1.12]	0.08	0.39	[0.09-1.63]	0.19
Pulmonary Fibrosis	0.96	[0.63-1.45]	0.84	1.56	[0.83-2.95]	0.17	0.50	[0.21-1.16]	0.11	0.64	[0.17-2.49]	0.52
Other	0.78	[0.46-1.32]	0.36	1.53	[0.81-2.90]	0.19	0.94	[0.42-2.10]	0.87	1.96	[0.72-5.32]	0.19
Type of transplantation												
Unilateral	1.00	(reference)	-	1.00	(reference)	-	1.00	(reference)	-	1.00	(reference)	-
Bilateral or combined organ	1.01	[0.74-1.39]	0.93	1.63	[1.08-2.46]	0.02	1.90	[0.91-4.00]	0.09	3.21	[1.24-8.33]	0.02
Immunosuppressive regimen												
CsA-based	1.00	(reference)	-	1.00	(reference)	-	1.00	(reference)	-	1.00	(reference)	-
Tac-based	0.93	[0.64-1.35]	0.69	0.82	[0.44-1.51]	0.51	0.83	[0.49-1.39]	0.47	0.83	[0.29-2.41]	0.73
Transplantation year	1.02	[0.99-1.05[	0.19	1.01	[0.96-1.06]	0.81	0.98	[0.93-1.03]	0.42	0.96	[0.86-1.07]	0.49

In analyses with pack years of smoking as a continuous variable, the HRs per 10 pack years of smoking for CKD-III and-IV were 1.19 [1.09–1.28], P<0.001 and 1.40 [1.20-1.62], P<0.001 (table 3). In the multivariate analyses this dose-dependent relation remained significant for both CKD-III (HR 1.25 [1.10–1.42], P<0.001) and CKD-IV (HR 1.46 [1.17–1.83], P<0.001) (table 3). The corresponding adjusted population attributable risks of former smoking (i.e. the CKD-rate reduction if all patients would have been never smokers) were 11.8% for CKD-III and 18.6% for CKD-IV.

In secondary analyses, the dose-dependent increase in renal risk persisted after exclusion of the never smokers from the analysis (table 4). Results of the analyses stratification by age (<47 and ≥47 years, population median) and diagnosis (COPD vs. non-COPD) are also displayed in table 4. As can be seen, hazard ratios were relatively high in patients < 47 years and in patients with diagnoses other than COPD. To investigate whether the risk was different between stratification groups, we tested for interaction between strata and pack years in the fully adjusted model. There was no significant interaction between age-strata and pack years for CKD stage III (P=0.92) and CKD stage IV (P=0.81). For diagnosis strata, there was a trend towards interaction for CKD stage III (P=0.18) and significant interaction for CKD stage IV (P=0.04). These data are consistent with pack years of smoking being a risk factor for CKD both in patients with COPD and patients without COPD, but highest risk in patients without COPD as primary diagnosis. As a total the results are consistent and point towards former smoking increasing the risk for CKD.

Table 4

Secondary Analysis		**CKD stage III** HR [95%CI]	*P-value*	**CKD stage IV** HR [95%CI]	*P-value*
Stratified by age					
< 47 years	Pack years*	1.30 [0.88-1.93]	0.19	2.31 [1.01-5.32]	0.05
> 47 years	Pack years*	1.21 [1.05-1.40]	0.008	1.36 [1.07-1.72]	0.04
Statified by diagnosis					
COPD	Pack years*	1.18 [1.02-1.38]	0.03	1.36 [1.07-1.72]	0.01
other than COPD	Pack years*	1.50 [1.11-2.01]	0.008	2.20 [1.17-3.97]	0.01
Former smokers only	Pack years*	1.23 [1.06-1.42]	0.005	1.42 [1.11-1.81]	0.005

***** per 10 pack years
All analyses are multivariate cause-specific hazards regression analyses for pack years and CKD-III (or greater) and CKD-IV (or greater)

We found that if analyses were repeated with CKD confirmed by a second value overall, cumulative incidences of CKD-III and CKD-IV after 5 years of follow-up were lower, with cumulative incidences of 57.8 and 10.4% for CKD-III and CKD IV respectively. The results of analyses of smoking as a risk factor for CKD remained essentially unchanged though, with HRs of 1.17 [1.03–1.33], P=0.02 and 1.63 [1.25-2.13], P<0.001 per 10 pack years of smoking for CKD-III and CKD-IV respectively in the fully adjusted model. Likewise, former smoking was dose-dependently associated with doubling of serum creatinine as renal endpoint, with a HR of 1.28 [1.10-1.49], P=0.002 per 10 pack years in the fully adjusted model. Lastly, we performed multivariate analyses with body-surface-area as a time-dependent variable, yielding HRs of 1.22 [1.08-1.39], P=0.002 for CKD-III and 1.48 [1.18-1.85], P=0.001 CKD-IV.

The overall cumulative incidence of all-cause mortality at 5 years after LTx was 34.6%, with a cumulative incidence of 32.9% never smokers vs. 37.2% in former smokers (P=0.4). In univariate Cox-regression analysis, former smokers' risk of mortality was similar to that of never smokers (HR = 0.99 [0.71–1.38], P=0.9). We found that the main causes of death were graft failure (34.2%, after mean 3.8±3.4 years) and malignancy (17.1%, after a mean of 5.0±4.0 years). Only a small proportion of patients died of cardiovascular causes (6.2%, after a mean of 4.6±4.6 years). Former smokers more often died of malignancy than never smokers (23% vs. 8.5% resp., P=0.03). There were no other significant differences in causes of death between former smokers and never smokers (Table 5).

Cause of death	Total N (%)	Never smokers N (%)	Former smokers N (%)	*P-value*
Peri-operative death	17 (11.8)	8 (13.6)	11 (12.6)	
Multi-organ failure	12 (8.3)	8 (13.6)	4 (4.6)	
Infection	19 (13.2)	7 (11.9)	12 (13.8)	
Cardiovascular	9 (6.3)	2 (3.4)	7 (8.0)	
Malignancy	25 (17.4)	5 (8.5)	20 (23)	0.03
Transplant failure	50 (34.7)	25 (42.4)	25 (28.7)	
Other	12 (8.3)	4 (6.8)	8 (9.2)	

Discussion

This study shows for the first time that former smoking is associated with increased risk of CKD-III and CKD-IV after LTx. We found the increased risk of former smoking on CKD to be independent of potential confounders, including baseline GFR, recipient age and pulmonary diagnosis. The association between former smoking and CKD was further supported by evidence of dose-dependency.

The difference in renal function between never and former smokers starts early after transplantation. Whereas both never smokers and former smokers had a steep initial decline in GFR, the initial decline in GFR was more pronounced in former smokers, and even more marked in former smokers with higher number of pack years. After the early rapid decrease in GFR, the rate of decline gradually leveled-off over time, but differences between groups clearly persisted.

Our results suggest that former smoking primes the kidneys for accelerated decline in GFR after LTx. The renal susceptibility to accelerated decline in GFR was not accounted for by adjustment for pre-transplant GFR. Nevertheless, it remains possible that former smokers have pre-existent renal damage. This is not necessarily captured by GFR as kidneys are known to have a certain amount of reserve capacity. This allows kidneys to maintain GFR at a certain level by compensating for damage and/or nephron loss. Consequently, substantial renal damage can be present without any measurable effect on GFR. In addition, it has been demonstrated that intra-renal vascular pathology (myointimal hyperplasia, arteriolar hyalinosis, glomerular sclerosis) in renal biopsies of former smokers is more prominent than in never smokers without an apparent difference in renal function (31). Thus, renal damage related to prior smoking can be present in the kidney after smoking cessation without being reflected in GFR. Interestingly, it has been observed that renal histological lesions (including myointimal hyperplasia and arteriolar hyalinosis) amplify the nephrotoxic effects of CNIs in renal transplant recipients (32). Accordingly, renal vascular lesions induced by prior smoking might well be involved in our observations that former smokers are more susceptible to post-transplantation CKD, despite that they no longer smoke. Further research will need to clarify whether these suggested mechanisms indeed play a role in the accelerated decline of GFR in former smokers.

Various mechanisms could underlie the persistence of smoking-associated damage after smoking cessation. Recently it was found that smoking-induced changes in epigenetics of blood platelets can persist for more than 10 years after smoking cessation, showing that distant effects of smoking can last for many years (33,34). These findings are corroborated by data of persisting increases in risk of smoking-associated conditions long after cessation of smoking, e.g. the

risk of lung cancer remains increased 15-fold in men and 9-fold in women for at least ten years after smoking cessation (35).
Our study is the first to identify former smoking as a risk factor for CKD in lung transplant recipients. The risk was not explained by clinical renal parameters, notwithstanding gold standard renal function measurements. This is of substantial clinical significance, as it means that reliable smoking history could aid in risk-assessment. In a general sense, the association between former smoking and the risk for CKD is very sparsely documented, and so far, data in populations with a high renal risk are lacking altogether.

We did not observe any relationship between former smoking and mortality after LTx, although former smokers more frequently died of malignancy. This would be consistent with the increased risk of malignancy in smokers in the general population. In the general population, smoking is furthermore associated with cardiovascular disease. However, no association between former smoking and cardiovascular death was observed in this study. Importantly, the chances of finding an association between former smoking and cardiovascular disease were small because LTx recipients generally are relatively young and thoroughly screened on cardiovascular status prior to transplantation. They are therefore unlikely to develop cardiovascular disease within several years. Moreover, despite the development of cardiovascular risk factors due to the use of immunosuppressants (hypertension, hyperlipidemia, hyperglycemia etc.), LTx recipients are more likely to die of other transplantation-related causes (such as transplant failure or infection). This is supported by the high mortality due to transplant failure and low numbers of cardiovascular death in our population.

Our study has several limitations. First, several differences in baseline characteristics existed between the never smokers and former smokers. The former smokers more often had pulmonary emphysema and were relatively old, whereas the never smokers often had cystic fibrosis, pulmonary fibrosis or pulmonary hypertension and were relatively young. To ensure that the results of our analyses were less likely to be confounded by these differences, we adjusted for recipient age, gender, baseline GFR, BMI, pulmonary diagnosis, type of transplantation, medication-regimen, hypertension, diabetes and transplantation year. We also performed several sensitivity analyses to check the consistency. Even so, the associations persisted.

In secondary analyses we found that in patients with diagnoses other than COPD packyears of former smoking was associated with highest risk for CKD. It should be noted that this group contains relatively many younger patients and patients with cystic fibrosis, whom differ in many aspects from patients with COPD (e.g. glomerular hyperfiltration making kidneys more susceptible to CNI-toxicity, higher prevalence of diabetes after LTx, higher prevalence of chronic rejection, higher doses of immunosuppression after LTx, all risk factors for CKD). It is possible that the difference between diagnosis strata is due to residual confounding, however, it is also possible that this reflects a difference in susceptibility of kidneys to the noxious effects of former smoking between the diagnosis groups.

In addition, it is hard to exclude that some patients have resumed smoking after LTx, despite very strong discouragement. Sobering data on smoking resumption after LTx have recently been published, showing that some 11% of patients had resumed smoking – without informing their doctors (15). Nevertheless, we believe it is unlikely that our conclusions are affected by an undetected subcohort that resumed smoking. First of all, others have reported much lower numbers of patients resuming smoking after LTx (16,36). Also, smoking resumption is more likely after an abstinence period of <6 months. Our center requires an abstinence period of at least 6 months before enlistment. Moreover, a random sample of patients from our population was recently tested for exhaled carbon monoxide levels and we found no evidence for smoking resumption. Last, whereas we cannot fully exclude smoking resumption in a minority of patients, it is unlikely that the presence of a small subcohort could lead to the clear dose-dependent effect of former smoking on CKD observed here in the whole cohort. Another limitation of the study is the lack of data on behavior, socio-economic status, presence of other co-morbid conditions and environmental factors. These parameters could be confounding the association between former smoking and CKD. While it would have been appropriate to adjust for these factors, such factors were not documented in the current study and could not be taken into account, resulting in potential unmeasured confounding.

A major strength of our analysis is the use of 125I-Iothalamate GFR measurements for baseline and follow-up. This method is considered a gold standard method for GFR measurement and provides a very reliable measurement (18). This measurement is superior especially in populations such as LTx, where long-term changes in muscle-mass confound creatinine-based renal function estimates (23). A comparison of this method with the more widely available eGFR calculations was published in an early subgroup (N=40) from this cohort in 2000 (23), showing that renal function loss is underestimated by creatinine-derived renal function estimates in LTx recipients. Another strength of this study is the availability of serial calcineurin inhibitor trough levels in never smokers and groups of former smokers. These levels were similar for never smokers and former smokers, indicating similar CNI exposure and making confounding by CNI toxicity unlikely. Furthermore, this study shows data on a large cohort of LTx recipients with a good representation of all kinds of LTx recipients with long follow-up, without loss to follow-up.

In conclusion, the number of pack years of prior cigarette smoking was dose-dependently associated with an increased risk of both stage-III and stage-IV CKD, but not all cause mortality in a large cohort of LTx patients, independent of potential confounders.

These findings are reason for concern. Waiting times for LTx have continued to increase due to expanding recipient criteria. Likewise donation criteria have also expanded. Patients as well as organs transplanted are generally in worse condition now than several years ago and LTx recipients are therefore at increased risk of complications after LTx, amongst which CKD (37). If the association of former smoking and CKD after LTx is confirmed in other studies, it would be relevant to gain further insight in the biology of this association. For the moment, it would seem prudent to consider former smokers at increased renal risk and avoid additional renal risks as much as possible.

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Chapter 4

Alcohol Consumption, New Onset of Diabetes After Transplantation and All- Cause Mortality in Renal Transplant Recipients

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Chapter 4

Abstract

Renal transplant recipients (RTR) are often advised to refrain from alcohol because of possible interaction with their immunosuppressive medication. While moderate alcohol consumption is associated with reduced risk of diabetes and mortality in the general population, this is unknown for RTR. Therefore, we investigated the association of alcohol consumption with new onset of diabetes after transplantation (NODAT), mortality and graft failure in RTR. RTR were investigated between 2001-2003. Alcohol consumption was assessed by self-report. Mortality and graft failure was recorded until May 2009. 600 RTR were studied (age 51±12 years, 55% men). Of these RTR, 48% were abstainers, 38% had light alcohol intake, 13% had moderate intake and 1% were heavy consumers. Moderate alcohol consumption was associated with a lower risk of developing NODAT over the follow-up period than was abstention (OR=0.36 [0.2-0.6], P=<0.001). During follow-up for 7.0 [6.2–7.5] years, 133 recipients died. In Cox-regression analyses moderate alcohol consumption was associated with lower mortality period than was abstention (HR=0.40[0.2-0.8], P=0.009). Adjustment for confounders, including age and smoking did not materially change this association. No association was found between alcohol consumption and graft failure. Moderate alcohol consumption is associated with low prevalence of NODAT and reduced risk for mortality in RTR, in line with findings in the general population. These findings refute the common advice to refrain from alcohol in RTR.

Introduction

Renal transplantation is the treatment of choice for patients with end-stage renal disease and allows freedom from lifestyle restrictions such as a strict diet (1). Short-term outcome after renal transplantation has improved substantially in the past decades, but long-term graft and patient survival have not improved in a similar manner (2). One main reason for persisting poor long-term outcome is premature death due to cardiovascular diseases (CVD), with incidence of CVD estimated to be 4-6 times higher in renal transplant recipients (RTR) than in the general population (3).

Interventions targeting modifiable risk factors such as hypertension, dyslipidemia, and physical activity, can improve outcome in RTR (4-6). Moderate alcohol consumption could be a behavioral lifestyle factor for lowering of risk of premature death, because numerous studies in the general population have demonstrated that moderate alcohol intake (1-3 units per day) is associated with reduced risk of diabetes, mortality and CVD compared to abstainers and sporadic users, while there is again an increased risk with intake above 3 units per day (7).

The KIDIGO Practice Guideline for the Care of Kidney Transplant Recipients does not mention specific alcohol restrictions for RTR (8). Accordingly, transplantation centers usually do not specifically advise RTR to refrain from post-transplantation alcohol use. Advice on the internet is, however, different. RTR are advised not to use alcohol for one year after transplantation, to avoid alcohol unless the doctor gives permission, or not to use any alcohol at all (9,10) .

One study showed that alcohol abuse is rare after kidney transplantation and that intake among RTR is generally low (11). No data are available on the relation between post-transplant alcohol use and long-term outcome.

Therefore, we aimed to investigate the prevalence and correlates of alcohol consumption in RTR in a large single-center cohort. Furthermore, we aimed to investigate whether alcohol consumption is associated with new onset of diabetes after transplantation (NODAT), all-cause mortality and graft survival in these patients.

Patients and Methods

Research Design and Subjects

The Institutional Review Board approved the study protocol (METc 2001/039), which was incorporated in the outpatient follow-up of the Groningen Renal Transplant Program. The outpatient follow-up constitutes a continuous surveillance system in which patients visit the outpatient clinic with declining frequency, in accordance with American Transplantation Society guidelines, i.e. ranging from twice a week immediately after hospital discharge to twice a year long-term after transplantation (55). All adult allograft recipients between August 2001 and July 2003 who survived with a functioning allograft beyond the first year after transplantation (1 year post transplant was considered baseline) were eligible to participate at their next visit to the outpatient clinic (index date). The group that did not sign informed consent was comparable with the group that did sign informed consent with respect to age, sex, body mass index, serum creatinine, creatinine clearance, and proteinuria. In patients with fever or other signs of infection (e.g. complaints of upper respiratory tract infection or urinary tract infection), baseline visits were postponed until symptoms had resolved. Patients with overt congestive heart failure and patients diagnosed with cancer other than cured skin cancer were not considered eligible for the study. A total of 606 out of 847 eligible renal transplant recipients signed written informed consent. Information on alcohol consumption was available in 600 patients. Alcoholism is considered a contraindication for transplantation in our center.

Endpoints of the study

The primary endpoints of this study were recipient mortality and graft failure. Graft failure was defined as a return to dialysis or re-transplantation. The continuous surveillance system of the outpatient program ensures up-to-date information on patient status and cause of death. We contacted general practitioners or referring nephrologists if the status of a patient was unknown. Mortality and graft loss were recorded until May 2009. Cause of death was obtained by linking the number of the death certificate to the primary cause of death as coded by a physician from the Central Bureau of Statistics. Causes of death were coded according to the International Classification of Diseases, 9th revision (ICD-9) (56). Cardiovascular death was defined as death in which the principal cause of death was cardiovascular in nature, using ICD-9 codes 410-447. There was no loss due to follow-up. NODAT was defined by fasting plasma glucose concentration was ≥7.0 mmol/l and/or use of anti-diabetic medication (57).

Renal Transplant Characteristics

Groningen Renal Transplant Database contains information on all renal transplantations performed at our center since 1968. Relevant transplant characteristics such as age, gender and date of transplantation were extracted from this database. Current medication was taken from the medical record. Smoking status and cardiovascular history were obtained using a self-report questionnaire. Cardiovascular disease history was considered positive if participants had a myocardial infarction (MI), transient ischemic attack (TIA) or cerebrovascular accident (CVA).

Measurements and definitions

Quantitative information on alcohol consumption was obtained by self-report questionnaire. Accordingly RTR were categorized into 4 groups of alcohol consumption (58): abstainers, light consumption (up to 10 gram per day), moderate consumption (10-30 gram per day) and high alcohol consumption (more than 30 gram per day). BMI, waist circumference and blood pressure were determined as described previously (56). MS was defined by the definition of the National Cholesterol Education Program Expert Panel (NCEP-ATPIII) as described earlier (56).

Blood was drawn after an overnight fasting period. We measured serum creatinine, serum triglycerides, total cholesterol, high-density lipoproteins-(HDL-)cholesterol, plasma glucose and HbA1c as described previously. Low-density lipoproteins (LDL) cholesterol was calculated using the Friedewald formula (59). Serum high sensitive C-reactive protein (hsCRP) was assessed with a high sensitivity CRP ELISA assay as described before (60). We performed uniform measurement of Gammaglutamyltransferase (GGT), asparaat-amino-transferase (ASAT), alanine-amino-transferase (ALAT), Alkaline phosphatase (AP) and lactaatdehydrogenase (LDH) activity in serum as described before.

Creatinine clearance was calculated from 24-hour urinary creatinine excretion and serum creatinine. Total urinary protein concentration was analyzed using the Biuret reaction (MEGA AU 510, Merck Diagnostica, Darmstadt, Germany) and proteinuria was defined as urinary protein excretion >0.5 g per 24 hours.

Statistical analysis

Data were analyzed with SPSS version 16.0 (SPSS Inc., Chicago, IL) and GraphPad Prism version 4.03 (GraphPad Software Inc., San Diego, CA). Normally distributed variables are expressed as mean ± standard deviation, whereas skewed distributed variables are given as median (25th-75th percentile), percentages were used to summarize categorical variables. Log-transformation was used for variables with a skewed distribution. Hazard ratio's (HR) are reported with 95% confidence interval [95% CI].

We analyzed recipient-related characteristics separately for the four categories of alcohol consumption. Student's *t*-test or Kruskal Wallis test was used to compare means for continuous variables and with Chi-square for categorical variables. Logistic regression analyses were used to analyze the association between alcohol consumption and risk for NODAT, for this analyses 17 RTR with pre-transplantation diabetes at baseline were excluded. To analyze whether alcohol consumption is associated with mortality and graft failure, we first performed Kaplan-Meier analyses with a Log-rank test. Univariate and multivariate Cox-regression analyses were performed to investigate whether alcohol consumption is independently associated with mortality and graft failure. Each baseline group of alcohol consumption was compared with the group of abstainers as reference group.

Results

A total of 600 RTR were studied (mean age 51±12 years, 55% men). Baseline characteristics according to alcohol consumption are shown in table 1. Of the 600 patients providing information on alcohol use, 288 (48%) were abstainers, 226 (38%) had light intake (< 10 gram per day), 78 (13%) had moderate intake (10-30 g per day) and 8 (1%) – all men – had high intake (>30 g per day).

Table 1 Characteristics in groups of alcohol consumption

	Abstainers N=288	<10 g per day N=226	10-30 g per day N=78	>30 g per day N=8	P-value
Recipient demographics					
Age, yr	53 ± 13	50 ± 12	52 ± 9	50 ± 11	0.1
Male, n (%)	105 (36)	226 (67)*	65(83) *	8 (100) *	<0.001
Being unfit to work, n (%)	78 (27)	62 (27)	21(27)	2 (25)	0.9

(Pre)transplant history					
Dialysis time, months	**29 [14-50]**	**28 [13-52]**	**21 [9-33] ***	**26 [20-38]**	**0.01**
Time between ntx and inclusion , yr	**6.0[3-12]**	**6.1 [3-11]**	**5.8 [3-11]**	**2.6 [1-9]**	**0.5**
Living donor, n (%)	**29 (10)**	**37 (16)**	**16 (21)**	**1 (13)**	**0.06**
Acute rejection [a], n (%)	**121 (42)**	**106 (47)**	**40(51)**	**5 (63)**	**0.3**
Cardiovascular Disease History					
Myocardial Infarction, n (%)	**27(9)**	**14 (6)**	**6(8)**	**1 (13)**	**0.5**
TIA/CVA, n (%)	**16(6)**	**15 (7)**	**2(3)**	**0 (0)**	**0.5**
Substance use					
Current smoking, n (%)	**54 (14)**	**55 (24)**	**20(26)**	**4 (50)**	**0.08**
Past smoking, n (%)	**105 (37)**	**102 (45)***	**43(55)***	**3 (38)**	**0.02**
History and current smoking, n (%)	**159 (55)**	**157(69)***	**63(81)***	**7(88) ***	**<0.001**
Body composition					
BMI, kg/m^2	**26±5**	**26±4**	**25±4**	**26±2**	**0.3**
Waist circumference, men (cm)	**100 ± 13**	**100 ± 13**	**99 ± 12**	**100 ± 10**	**0.9**
Waist circumference, women (cm)	**95 ± 15**	**94±15**	**86±10**	**-**	**0.1**
Blood pressure					
Systolic BP, mmHg	**153 ± 24**	**153 ± 21**	**152± 22**	**166 ± 17**	**0.5**

Diastolic BP, mmHg	89 ± 10	90 ± 9	90± 9	104 ± 5*	0.001
Use of antihypertensive drugs, n (%)	246 (85)	201 (89)	69(88)	8 (100)	0.4
Lipids					
Total cholesterol, mmol/l	5.5 ± 1.0	5.6 ± 1.2	5.7 ± 1.0	5.8 ± 0.7	0.5
HDL cholesterol, mmol/l	1.1 ±0.2	1.1 ±0.3	1.1 ±0.3	1.3 ±0.6	0.2
LDL cholesterol, mmol/l	3.5 ±0.9	3.6 ± 1.1	3.7 ± 1.0	3.3 ± 0.7	0.07
Triglycerides, mmol/l	2.0 [1.4-2.8]	1.9 [1.3-2.5]	1.8 [1.4-2.3]	2.4 [1.8-3.6]	0.1
Statins, n (%)	145 (50)	102 (45)	41(53)	8 (100)*	0.02
Glucose homeostasis					
Glucose, mmol/l	4.9±1.4	4.8 ±1.5	4.8 ±1.1	4.7 ±0.5	0.9
Insulin, (µmol/L)	12.0 [9-17]	10.6 [8-15]*	9.1 [7-13]*	9.0 [6-15]	0.001
Hba1c, %	6.6±1.1	6.5 ± 1.0	6.5 ± 1.1	6.3 ± 0.8	0.5
NODAT, n (%)	73 (25)	42(19)	9(12)*	1(13)	0.03
Pre transplant diabetes, n (%)	13(5)	12(5)	3(4)	0(0)	0.8
Metabolic Syndrome n, (%)	196 (68)	145 (64)	40(51)*	4 (50)	0.04
Inflammation					
CRP, mg/l	2.2 [1-5]	1.9 [1-5]	1.8 [1-4]	1.0 [0-3]	0.2
Immunosuppressive medications					
Daily prednisolone dose, mg/day	10.0 [8-10]	10.0 [8-10]	10.0 [8-10]	10.0 [8-10]	0.3
Calcineurin inhibitors, n (%)	224 (78)	183 (81)	56(72)	7 (88)	0.3

Proliferation inhibitors, n (%)	**205 (71)**	**170 (75)**	**62(79)**	**7 (88)**	**0.3**
Renal allograft function					
Serum creatinine, Men (μmol/l)	**137 [119-171]**	**146 [125-177]**	**144 [124-182]**	**166 [145-214]***	**0.03**
Serum creatinine, Women (μmol/l)	**119 [99-149]**	**127 [97-156]**	**127 [106-155]**	**-**	**0.7**
Creatinine clearance, ml/min	**60 [45-75]**	**60 [47-76]**	**66 [51-81]**	**58 [45-76]**	**0.2**
Proteinuria, g/24h	**0.2 [0.0-0.5]**	**0.3 [0.1-0.6]**	**0.3 [0.1-0.5]**	**0.4 [0.2-0.8]**	**0.2**
Liver function					
GGT, U/L Men	**25 [17-38]**	**24 [19-38]**	**26 [18-42]**	**33 [22-49]**	**0.4**
GGT, U/L Women	**24 [17-40]**	**23 [16-35]**	**22 [20-34]**	**-**	**0.6**
ASAT, U/L	**23 [19-28]**	**22 [19-26]**	**23 [18-27]**	**21 [18-29]**	**0.8**
ALAT, U/L	**17 [13-23]**	**29 [14-25]**	**19 [14-26]**	**22 [14-26]**	**0.8**
AP, U/L	**74 [60-96]**	**71[55-94]**	**72[57-84]**	**65[50-102]**	**0.4**
LDH, U/L	**260 [233-310]**	**261 [224-297]**	**250 [228-286]**	**266 [228-290]**	**0.4**

Data are represented as mean ± SD, or median [95% CI]. Differences were tested by *t*-test or Kruskal Wallis test for continuous variables and with Chi-square for categorical variables. *Group significantly different from reference group (abstainers), P <0.05. Time between ntx and inclusion, yr, time between transplantation and inclusion, years; [a] Acute rejection treatment with high dose corticosteroids. TIA, transient ischemic attack; CVA, cerebrovascular accident; BMI, body mass index; BP, blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; NODAT, new onset of diabetes after transplantation; CRP, C-reactive protein; GGT; gammaglutamyltransferase; ASAT, asparaat-amino-transferase; ALAT, alanine-amino-transferase; AP; alkaline phosphatase LDH, lactaatdehydrogenase

Moderate alcohol consumers were more likely to have a shorter dialysis time, a living donor and higher LDL-cholesterol. Alcohol consumption was positively associated with male gender, smoking and past smoking, diastolic blood pressure and use of statins and serum creatinine concentrations in men. There was an inverse association between alcohol consumption and prevalence of Metabolic syndrome (MS), fasting insulin concentrations and prevalence of NODAT. Total prevalence NODAT was 125 (21%). Results of univariate and multivariate logistic regression analyses for alcohol consumption and risk for NODAT are shown in table 2. Moderate alcohol consumption was strongly associated with low prevalence of NODAT (Odds ratio= 0.37 [0.17-0.78], P=0.008). A total of 385 RTR (64%) fulfilled the criteria of MS. Prevalence of MS decreased according to increasing alcohol consumption (P= 0.04).

Table 2. Logistic regression analyses for NODAT according to groups of alcohol consumption

	Alcohol consumption						
	Abstainers N=278	<10 g per day N=219		10-30 g per day N=78		>30 g per day N=8	
	Reference	HR [95%CI]	P-value	HR [95%CI]	P-value	HR [95%CI]	P-value
Model 1	1.0	0.67 [0.43-1.02]	0.06	0.37 [0.17-0.78]	0.008	0.40 [0.05-3.31]	0.4
Model 2	1.0	0.76 [0.48-1.21]	0.25	0.41 [0.19-0.90]	0.026	0.51 [0.06-4.39]	0.5
Model 3	1.0	0.78 [0.49-1.23]	0.30	0.42 [0.19-0.93]	0.03	0.55 [0.06-4.70]	0.6
Model 4	1.0	0.72 [0.44-1.17]	0.20	0.43 [0.19-0.97]	0.04	0.57 [0.07-4.91]	0.6

Model 1: Crude model Model 2: model 1 + adjustments for age and sex
Model 3: model 2 + current smoking and past smoking Model 4: model 3 + BMI

During follow-up for 7.0 [6.2 – 7.5] years, 133 RTR died and 52 RTR suffered graft failure necessitating their return to dialysis. In the group with moderate alcohol consumption 9 (12%) RTR died during follow-up, whereas this number was 75 (26%) for the group with no consumption, 47 (21%) in the group with light consumption (Log-rank test P=0.02, fig. 1a), and 2 (25%) in the group with high alcohol consumption.

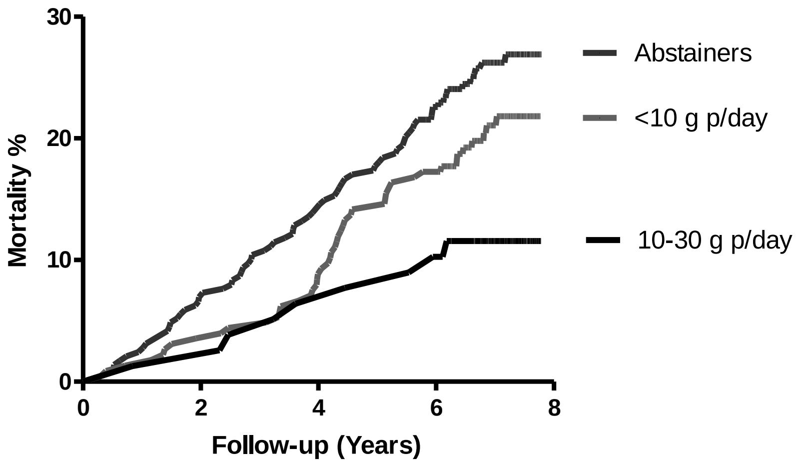

Figure 1a. Kaplan Meier curves of mortality to alcohol consumption, tested with Log-rank test (P= 0.02).

Results of univariate and multivariate Cox-regression analyses for associations of alcohol consumption with mortality are presented in table 3. Moderate alcohol consumption was strongly associated with reduced risk for mortality (model 1) in univariate analyses (HR=0.40[0.2-0.8], P=0.009). Upon multivariate analyses, these associations weakened after adjustment for age and sex (model 2). Adjustment for current smoking and past smoking strengthened the association (model 3). Further adjustments for BMI, use of statins and LDL-cholesterol (model 4) living donor and dialysis time (model 5) and serum creatinine (model 6) did not materially changed the association.

Table 3. Cox regression analyses for mortality according to groups of alcohol consumption

	Alcohol consumption						
	Abstainers N=288	<10 g per day N=226		10-30 g per day N=78		>30 g per day N=8	
	Reference	HR [95%CI]	P-value	HR [95%CI]	P-value	HR [95%CI]	P-value
Model 1	1.0	0.76 [0.52-1.10]	0.15	0.40 [0.20-0.79]	0.009	0.93 [0.25-3.77]	0.9
Model 2	1.0	0.85 [0.58-1.25]	0.42	0.44 [0.22-0.91]	0.026	1.42 [0.34-5.93]	0.6
Model 3	1.0	0.74 [0.50-1.08]	0.12	0.38 [0.19-0.78]	0.008	1.17 [0.28-4.97]	0.8
Model 4	1.0	0.74 [0.50-1.09]	0.15	0.39 [0.19-0.80]	0.010	1.20 [0.28-5.08]	0.8
Model 5	1.0	0.75 [0.51-1.10]	0.14	0.42 [0.21-0.88]	0.021	1.21 [0.29-5.12]	0.8
Model 6	1.0	0.74 [0.50-1.09]	0.23	0.44 [0.21-0.90]	0.025	1.19 [0.28-5.05]	0.8

Model 1: Crude model
Model 2: model 1 + adjustments for age and sex
Model 3: model 2 + current smoking and past smoking
Model 4: model 3 + BMI, statin use, and LDL-cholesterol
Model 5: model 4 + living donor and dialysis time
Model 6: model 5 + serum creatinine

In the group with moderate alcohol consumption 9 (12%) RTR developed graft failure during follow-up, whereas this number was 23 (8%) for the group of no alcohol consumption and 19 (8%) for the group with light alcohol consumption (Log-rank test P=0.6, fig.1b). In the group with high alcohol consumption 1 (12.5%) RTR developed graft failure. In a univariate Cox-regression analysis moderate alcohol intake was not associated with graft failure (HR =1.44 [0.7-3.1], P=0.4).

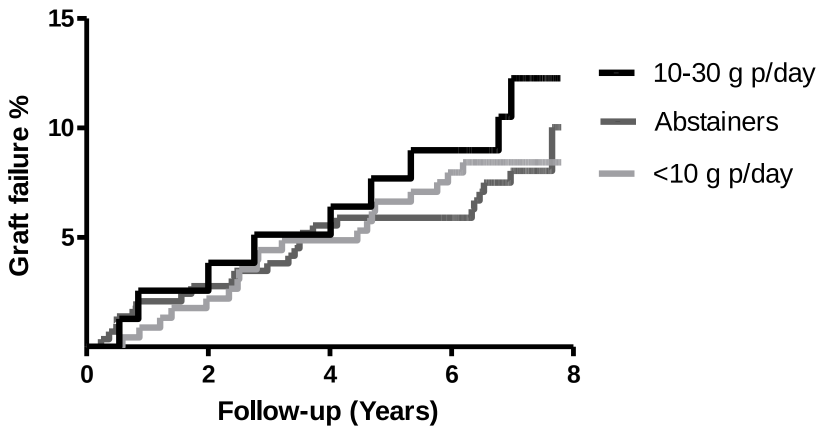

Figure 1b. Kaplan Meier curves of graft loss according to alcohol consumption, tested with Log-rank test (P= 0.6).

Discussion

This is the first study to report on habitual alcohol consumption, NODAT and all cause mortality in RTR. It suggests that moderate alcohol consumption has protective effects in RTR that correspond to those in the general population, despite differences in disease burden and medication use. In particular, moderate alcohol consumption is associated with lower risk for NODAT and all-cause mortality. No association was found between alcohol consumption and graft loss.

The prevalence of alcohol consumption in our study population (52%) is similar to the prevalence and severity of alcohol consumption in RTR reported by Fiertz (11), and lower than in the general population, where alcohol consumption is approximately 89% in men and 74% in women (12). This suggests that RTR limit alcohol consumption, either spontaneously or as a consequence of advice. In agreement with guidelines, we do not actively advise

RTR to refrain from post-transplantation alcohol use in our center. On the other hand, advice on the internet does (9,10) and there may be a general tendency for patients and physicians to adhere to this. Reasons for advising against alcohol consumption could be several. Due to the immunosuppressive regime, transplant doctors are often cautious with advice on alcohol consumption. It may for instance be thought that great amounts of alcohol interfere with liver metabolism, in addition to calcineurin inhibitors, such as cyclosporine, stressing the liver. Another reason may be the data provided by the only other prospective study of the effects of alcohol consumption in RTR. In this study, Gueye et al. analyzed renal graft and patient survival in 425 RTR with alcohol dependence before transplantation compared to 60 523 RTR without alcohol dependence (13), rather than average daily intake after transplantation, as we did. They concluded that alcohol dependence before transplantation is a risk factor for renal graft failure and death and advised against use of alcohol in RTR. Our study suggests that average daily intake of alcohol after transplantation cannot be compared to alcohol dependence before transplantation.

New onset diabetes is a common complication of transplantation and a major risk factor for graft failure and mortality in RTR (14,15). Traditional risk factors like obesity, diabetes mellitus, dyslipidemia and hypertension are often seen in transplantation patients and cluster in the MS (16). Our data confirm this with a prevalence MS of 64%.

While there is no other literature addressing alcohol consumption and long-term outcome in RTR, the inverse association between alcohol consumption and cardiovascular diseases in the general population is well documented. Moderate alcohol consumption is associated with a decreased risk for myocardial infarction (17,18), heart failure (19,20) and ischemic stroke (21-23). It also reduces the risk of myocardial and all-cause mortality (24-28). Moderate alcohol consumption is also associated with a decreased risk of developing type 2 diabetes (29,30). Our study shows that moderate alcohol consumption is associated with 60% decreased risk for all cause mortality, compared to the group of abstainers. The risk reduction of moderate alcohol consumption in the general population is estimated to be between 20-30%. The magnitude of risk reduction seems to be higher in RTR. This is in line with a previous study which showed larger risk reductions for those with an increased cardiovascular risk (31).

There is little known about the influence of alcohol consumption on renal function and graft survival and results are inconsistent. Studies in general population cohorts suggest no adverse effects of alcohol consumption and even a protective effect of alcohol consumption on renal function (32,33) has been described. Other studies suggest a negative link between alcohol intake and kidney function. In an Australian population-based study all alcohol intake of ≥30 g/day was independently associated with an increased risk of albuminuria (34). Another population based study (35) showed a 4-fold increased risk of end-stage renal disease in subjects who consumed ≥2 units alcohol per day. It has been argued that the pressor effect of alcohol might increase the risk for renal disease (36). Indeed, we found evidence for a decreased renal function in the group with high alcohol consumption in our study. Serum creatinine was significantly higher in the group with high alcohol consumption and there was a trend for a lower creatinine clearance and higher proteinuria with high alcohol consumption. Systolic blood pressure was also significantly higher in the RTR with high alcohol consumption. Although we found no significant association between alcohol consumption and graft failure, this is possibly the consequence of the group being very small, increased systolic blood pressure and increased urinary protein excretion are consistent with an adverse effect of heavy alcohol consumption on graft function.

We anticipated that a potential association of moderate alcohol consumption with mortality could be confounded by other risk factors for mortality or a healthier lifestyle. We found, however that the association between alcohol consumption and all-cause mortality was not materially affected by adjustments for current smoking and past smoking, BMI and LDL-cholesterol. Tobacco use is an important confounder in the relationship between alcohol and mortality.

Tobacco use is often greater in people who drink alcohol (37). History of smoking and current smoking in our population increased from 159 (55%) to 157 (69%), 63 (81%) and 7 (88%) according to increasing alcohol consumption (P< 0.001). Studies showed that smoking has a major negative impact on mortality and graft survival in RTR (38,39). The strengthening of the association after adjustment for smoking (table 2, model 3) suggests that alcohol consumption has a stronger protective effect if it is not combined with smoking. It has been argued that moderate drinkers may represent a relatively healthy subpopulation, with a general healthier lifestyle, related to the fact that people in a poor overall condition are less likely to consume alcohol, which may explain a small proportion of the effect of alcohol consumption on mortality (40). Various studies have removed the effect of so called ‘sick quitters’ by taking lifelong abstainers as reference group and looked at the effects independent of other healthy lifestyle factors, showing that moderate alcohol consumption is causally related to a lower risk for cardiovascular diseases (41).

There is substantial evidence to support a causal relationship between alcohol consumption and CVD in the general population (42). The protective effect of moderate alcohol consumption can be explained by several mechanisms. An important way by which alcohol consumption can modulate the CVD risk is by changes in lipid profile. Moderate alcohol consumption is associated with higher HDL- cholesterol and apolipoprotein A1 levels (43,44). Alcohol also has been shown to decrease platelet aggregation and lower concentrations of plasma fibrinogen (45,46). Another important mechanism that could modulate CVD risk is by lowering insulin resistance. Moderate alcohol consumption increases the insulin sensitivity and thereby lowers the risk for developing diabetes (29,47,48). The same mechanisms could be relevant to reduce mortality risk in RTR. Our results indicate that RTR with moderate alcohol consumption had lower insulin levels and lower prevalence of diabetes, implicating a possible effect of alcohol on insulin sensitivity. Moderate consumers also had higher levels of LDL- cholesterol and lower triglycerides (borderline significant). The

small differences in lipid levels could partly be explained by the frequent use of statins among our RTR.
The strength of our study is its prospective design. RTR in this study were closely monitored by regular check-ups in our clinic, which give complete information on patient status. Some limitations of our study warrant consideration. First, this study relied on self-reported data. Recall bias and social desirability bias are unfortunately unavoidable and could influence internal validation. Self-reported alcohol intake is generally under reported (49). However, studies have found that questionnaires provide enough validity for ranking participants on alcohol consumption (50). Second, we acquired information on average daily intake with no details on drinking patterns. The cardio protective effect occurs in regular drinkers and not in binge and irregular heavy drinkers (51). Moreover, our study used a single measurement of alcohol consumption and did not take into account any changes in alcohol consumption over time. Finally, it was a single center study in a predominantly Caucasian population. In our study prevalence of diabetes and body mass index were relatively low. The contribution of diabetes mellitus to end-stage renal disease in the Netherlands as compared its surrounding countries is relatively low (52-54). This may at least in part explain the low prevalence of diabetes in our study. Similarly, the relatively low BMI may be a reflection of the relatively low contribution of type 2 diabetes to end-stage renal disease in our population.

This study shows that moderate alcohol consumption is inversely associated with NODAT and all cause mortality in RTR. So, in contrast to common belief, drinking moderate amounts of alcohol does not appear to be detrimental and may be protective against diabetes and mortality in RTR, similar to the general population, despite differences in disease burden and medication use. Further research is needed to confirm these findings and to investigate the relationship between alcohol consumption and graft survival.

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Chapter 5

Renoprotective Effects of Long Term Oral Nicotine in a Rat Model of Spontaneous Proteinuria[1]

Pramod K Agarwal , Jacob van den Born , Harry van Goor , Gerjan Navis , PhD, Rijk OB Gans and Stephan JL Bakker

[1] This article is accompanied by an editorial.

Chapter 5

Abstract
Many proteinuric renal conditions are accompanied by renal inflammation. Nicotine is known to have anti-inflammatory properties, and is used in oral form to quit smoking. A potential anti-inflammatory role of nicotine in proteinuric renal diseases has not been investigated to date. We therefore evaluated the effects of oral nicotine in a rat model of proteinuria-induced renal inflammation. We used a well-established model of adult (24 wk of age) male Munich-Wistar-Frömter rats. Animals were given 3 different physiological doses of Nicotine in drinking water for 28 weeks, till 52 wk of age (long term). A group without nicotine served as parallel control. At 52 weeks of age, the control group had 2.1 times reduction in creatinine clearance, 3.2 times increase in urinary protein excretion, increased focal glomerulosclerosis (FGS) score, glomerular desmin deposition and podocytopathy (podocin loss) and higher accumulation of macrophages and myofibroblasts compared to 24 wk animals. Oral treatment with nicotine dose-dependently preserved renal function and halted proteinuria progression, which were independent of blood pressure reduction. Oral nicotine also reduced FGS, desmin deposition and podocin loss and density of renal macrophages and myofibroblasts. Nicotine also reduced the level of gene expression of the renal inflammatory markers MCP-1 and VCAM-1. In conclusion, long term oral nicotine preserved kidney function, reduced proteinuria, reduced renal inflammation and protected progression of renal structural damage in a rat model of proteinuria. We further suggest evaluating nicotine as a potential additional therapeutic option for treating proteinuric kidney diseases.

Nicotine is Renoprotective

Introduction

Reduction of blood pressure and proteinuria currently are the cornerstone of renoprotective intervention. However, despite correction of hypertension and reduction of proteinuria, renoprotection is often incomplete and many patients progress towards renal failure. Even forced down titration of proteinuria by dual Renin Aldosterone Angiotensin System (RAAS) intervention (ONTARGET trial) or Angiotensin Converting Enzyme (ACE)-inhibition so far was not successful to improve renal outcome (1). Rather, under very low salt conditions such stringent measures may worsen outcome (2). This indicates the need for additional treatment modalities for renoprotection: not only in trying to reduce proteinuria even further, but also in reducing the harmful effects downstream of proteinuria. Evidence for this approach came amongst others from a bicarbonate study in hypertensive nephropathy patients, showing that oral bicarbonate is an effective kidney-protective measure in adjunction to blood pressure control and ACE-inhibition, most likely through reduction of tubulo-interstitial injury (3,3). Similarly combination of vitamin D with AT1 receptor blocker reduces harmful effects of diabetic nephropathy (4). It is well recognized that proteinuria leads to renal inflammation and fibrosis, most likely via tubular activation by filtered proteins, leading to the production of a cascade of mediators by tubular cells, including chemo- attractants for leukocytes such as MCP-1 (5). Consequence is an influx of monocytes and macrophages, which mediate renal inflammation and fibrosis (6) .

Renal inflammation is seen in many proteinuric renal diseases. Chronic renal inflammation is related to progressive decline in kidney function, glomerulosclerosis, interstitial damage (7) and resistance to renoprotective therapy (8). Recruited monocytes and macrophages orchestrate inflammation related damage. Reduction of inflammation by immunosuppressive drugs has been shown to slow down renal dysfunction with reduction in glomerulosclerosis and interstitial inflammation (9-12).

Smoking is a risk factor of development and progression of diabetic nephropathy, a risk factor for progression of chronic kidney disease to end stage kidney disease (13,14) and also a risk factor for graft failure in renal transplant patients (13). Cigarette smoke contains many compounds of hydrophilic, lipophilic and ambiphilic nature, which together are supposed to mediate cigarette smoking related renal damage. Whether nicotine contributes to harmful effects of cigarette smoke on renal function is not clear. Nicotine is a major constituent of cigarette smoke and used widely in oral form to quit smoking (15-20). Previous studies have shown nicotine to be clinically beneficial in many diseases in which an inflammatory component plays a role (21). Notably, nicotine has been shown beneficial in ulcerative colitis (22), sepsis (23), hypersensitivity penumonitis (24), renal ischemic reperfusion injury (25) and experimental type 1 diabetes (26).
Nicotine exerts its anti-inflammatory effects via α-7 nicotinic Acetylcholine Receptor (α-7nAchR) (27-29) which are present on macrophages and peritubular capillaries in kidneys (25,30). Thus, nicotine might have renoprotective potential by its anti-inflammatory properties, but this hypothesis has not been tested so far in chronic proteinuric settings. In this study, we therefore evaluated the effects of long term oral nicotine on renal function and inflammation in Munich-Wistar-Fromter (MWF) rats, which develop spontaneous, proteinuria-induced renal inflammation (31-34).

We found that long term oral nicotine slowed down the rise in proteinuria, slowed down loss of kidney function and reduced glomerular injury and inflammatory cellular infiltration in a rat model of spontaneous proteinuria.

Methods

Animals and housing

Inbred 20 weeks male Munich-Wistar-Fromter rats (n=46) were obtained from Harlan, USA. All animals were housed in temperature controlled 12h light/12h dark rooms and had free access to food and drinking water. All animals received human care in compliance with the Principles of Laboratory Animal Care (NIH Publication no. 85-23, revised 1996) and the protocol was approved by University Medical Center Groningen Animal Ethical Committee.

Experimental setup

After acclimatization for 2 weeks, animals were trained for tail cuff blood pressure measurement (CODA 6, Kent Scientific Corporation, USA) daily for another 2 weeks. At 24 week of age baseline blood pressure was measured, venous blood was withdrawn and 24h urine was collected in metabolic cages. For baseline histology six rats were sacrificed at the age of 24 weeks. The other animals were randomly assigned to four different groups. Rats were given either of 3 different concentrations 20 mg/l (n=10; N20), 60 mg/l (n=10; N60) and 100 mg/l (n=10; N100) of nicotine mixed with 0.5% sodium saccharin as sweetening agent *ad-libitum* in drinking water. Control group (n=10; CON) only received water sweetened with 0.5% sodium saccharin. Treatment continued for 28 weeks (until 52 weeks of age). One animal in N60 was sacrificed prematurely because of tooth malformation due to which the animal was unable to eat and lost weight. One animal in N20 was sacrificed because of a bone tumor. The tumor formation is unlikely to be related to nicotine since it was a sporadic case which might be related to aging. Both the animals were excluded from all analyses.

Nicotine intake and Cotinine measurement

Amount of nicotine intake per day is equal to 24h water intake of individual rat in metabolic cages X concentration of nicotine in water. Cumulative dose of nicotine intake was calculated by calculating the area under curve of nicotine intake over 28 treatment weeks. Cotinine is a major and stable metabolite of nicotine and used to quantify smoking (35). Cotinine was measured in plasma to quantify the amount of nicotine by Immunometeric Assay IMMULITE ^{Tm} 2000 from Siemens. The range of measurement was >10 ng/ml and <500 ng/ml.

Clinico-pathological parameters
Blood pressure was measured in trained awake animals with tail-cuff method. 24h urine and venous blood were collected at every 4 weeks until the end of the study. 24h urine were collected and stored at -20 °C until analyses were performed. Venous blood was collected with heparin and stored in -80 °C. Urinary and plasma creatinine and urinary protein were measured by multi-test analyzer system (Roche Modular; F.Hoffmann-La Roche Ltd, Basel, Switzerland). Creatinine clearance was calculated from 24h urinary volume, plasma and urinary creatinine (36).

Sacrifice and organs
Animals were sacrificed under isofluran-induced general anesthesia by cutting the diaphragm. Animals were flushed with 0.9% NaCl, kidneys were harvested, and weights were measured and were partly stored in -80 °C and partly fixed in 4% formalin followed by embedment in paraffin.

Morphological analysis
4 µm thick formalin fixed paraffin sections were deparaffinized and stained for periodic acid Schiff (PAS) for quantification of focal segmental glomerulosclerosis (GS). GS was semi quantitatively scored in a blinded fashion by determining the level of mesangial expansion and focal adhesion in each quadrant in a glomerulus and expressed on a scale from 0 to 4. If 25% of the glomerulus was affected, it was scored as 1, 50% as 2, 75% as 3 and 100% as 4. In total, 50 glomeruli per kidney were analyzed, and the total GS score was calculated by multiplying the score by the percentage of glomeruli with the same GS score. The sum of these scores gives the total GS score from 0 to 200 (36).

Immunohistochemistry

Deparaffinized and rehydrated sections (4 µm) were subjected to heat-induced antigen retrieval by overnight incubation in a 0.1 M Tris/HCl buffer (pH 9.0) at 80°C. Endogenous peroxidase was blocked with 0.3% H_2O_2 in phosphate-buffered saline (PBS) for 30 min and sections were incubated with ED1 antibody to visualize monocytes/macrophages (Serotec, Oxford, UK) or anti smooth muscle actin (A5228, Sigma-Aldrich, Zwijndrecht, The Netherlands) to show myofibroblasts, anti-desmin antibody to show podocyte injury (Novus Biologicals, USA) and anti-podocin antibody to show podocytopathy (Sigma, USA) for 60 min at room temperature. Binding of the antibody was detected using sequential incubations with horseradish peroxidase (HRP)-labelled rabbit anti-mouse and HRP-labelled Swine anti-rabbit antibodies (DAKO); both for 30 min. Peroxidase activity was developed using 3-Amino-9-Ethylcarbazole (AEC) for 15 min. Sections were counterstained with haematoxylin. Monocyte/macrophages influx and myofibroblasts were quantified in 50 cortical and outer medullary areas using image processing and analysis programme (ImageJ) magnified 200 times in a standardized and blinded fashion. Desmin and podocin positivity was quantified in a similar fashion. The results are presented as percentage of positively stained area relative to total area measured. Arteries were excluded from analysis.

Quantitative real time RT-PCR (qPCR)

Total RNA was extracted from 25-30 mg of frozen rat kidney tissue using RNeasy mini kit from QIAGEN. cDNA was synthesized using 1 µg of RNA by QuantiTect Reverse Trasncriptase kit (QIAGEN, catalogue number 205313). PCRs were performed in a 10 µl reaction containing 6.6 ng of RNA and using 2X Sensimix CYBR Green mastermix kit (QIAGEN catalogue number QT 650). qPCR was performed in C1000 Thermal cycle from Biorad. On demand primers for GAPDH, MCP-1, VCAM-1 were obtained from QIAGEN. Results are expressed relative to CON after sacrifice at 52 wk of age after normalizing for GAPDH a house keeping gene.

Statistical analyses

Statistical analysis was performed using Spss 14. Area under curve and figures were made by GraphPad Prism (version 4.00; GraphPad Software, Inc., San Diego, CA). Parametric values are expressed as mean (standard error of the mean). Non parametric values are expressed as median (Inter Quartile Range) and were log transformed for further analysis. ANOVA with post-hoc tests according Tukey for comparison of treatment groups and controls at 52 weeks and general linear model for repeated measurements for comparison of the time course of proteinuria between treatment groups were used. $P < 0.05$ was considered statistically significant.

Results

Nicotine intake and clinico-pathological parameters

Parameters concerning nicotine intake and clinico-pathological markers are shown in table 1. Cumulative nicotine intake was calculated from concentrations added to drinking water and volume of water intake. Nicotine intake was significantly different among the three treatment groups and there was no nicotine intake in the control (CON) group. Analogous, there was a dose-dependent difference in plasma cotinine concentrations between the treatment groups and no detectable plasma cotinine in the CON group. CON group had significantly higher plasma triglyceride compared to N60 and N100. There were no differences in body weight, water intake and systolic blood pressure among the groups during the experiment.

Clinicopathological characteristics at baseline and after 28 weeks of oral nicotine treatment is given in table 1.

Table 1

	Baseline	After 28 weeks of treatment			
	(n=46)	CON (n=10)	N20 (n=9)	N60 (n=9)	N100 (n=10)
Body weight (g)	339 (3)	385 (10)	366 (10)	367 (8)	375 (7)
Water intake (ml/24h)	21 [14-27]	12 [7-15]	9 [7-15]	14 [8-27]	9 [8-13]
Cumulative nicotine intake (mg)	0 (0)	0 (0)	9682 (1141)***	17310 (1010)***&	32996 (2707)***&$
Plasma cotinine (ng/ml)	0	0	139±16**	357±27**&	>500**&$
24h urine output (ml/24h)	17 [14-23]	16 [10-20]	11 [9-14]	10 [8-11]	10 [7-13]
Systolic blood pressure (mm Hg)	177 (2)	181 (6)	180 (6)	176 (5)	174 (5)
Plasma creatinine (μmol/l)	26 (1)	50 (7)	37 (5)	31 (1)*	32 (2)*
Plasma glucose (mmol/L)	7.6 (0.4)	7.0 (0.4)	7.5 (0.4)	7.4 (0.2)	7.0 (0.1)
Plasma cholesterol (mmol/L)	2.5 (0.1)	5.0 (0.3)	4.0 (0.2)	4.0 (0.3)	4.0 (0.3)
Plasma triglyceride (mg/dl)	N/A	233 (14)	190 (26)	160 (30)*	164 (22)*
Plasma urea (mmol/l)	9 (0.3)	27 (2.0)	23 (3.0)	22 (1.0)	22 (2.0)
Kidney weight/100gm body weight	0.7 (0.01)	0.7 (0.03)	0.7 (0.03)	0.7 (0.01)	0.7 (0.02)

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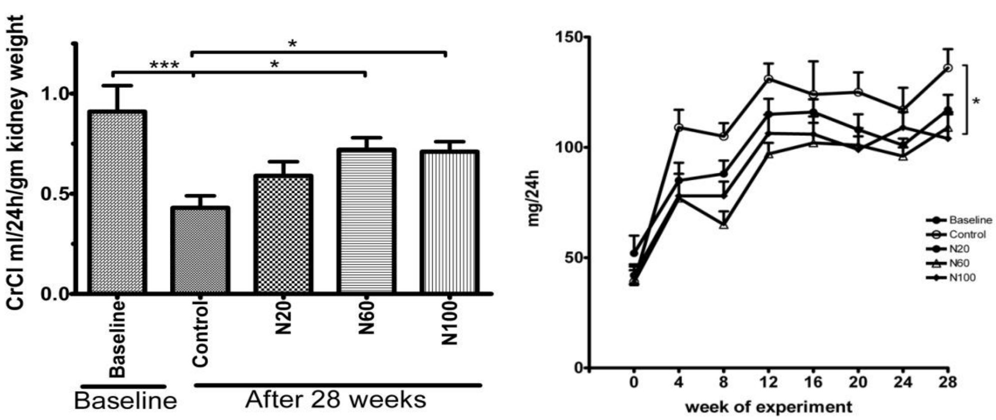
Abbreviations: CON: MWF without nicotine; N20: MWF rats treated with 20mg/l nicotine; N60: MWF rats treated with 60mg/l nicotine; N100: MWF rats treated with 100mg/l nicotine. *p <0.05 vs control **p<0.01 vs control *** p<0.001 vs control ; & p<0.001 vs N20; $ p<0.001 vs N60. Parametrical values are given as mean (SEM) whereas non parametrical values are given as median [inter quartile range].

Renal parameters

Plasma creatinine increased from 26±2 μmol/l at 24 weeks of age in the baseline group to 50±7 μmol/l at 52 weeks of age after 28 weeks of placebo with sodium saccharin (CON) (table 1, p=0.003). After 28 weeks of nicotine treatment, groups N60 and N100 had significantly lower serum creatinine

(31±1 μmol/l and 32±2 μmol/l, both $p<0.05$) than the CON group. To certify that
observed differences are the consequence of changes in kidney function and not changes in muscle mass, creatinine clearance was calculated. Nicotine treatment improved kidney function dose-dependently compared to CON ($p=0.003$) (figure 1A). Urinary protein excretion (UPE) increased spontaneously in MWF rats during the experiment, with values of 38 (22-57) mg/24h and 140
(105-159) in the CON group at the end of the experiment ($p<0.001$). Nicotine treatment for 28 weeks reduced UPE compared to CON ($p=0.03$ all nicotine groups vs CON) (figure 1B). We further calculated the percentage reduction of 24h UPE by nicotine treatment at end of the experiment ie after 28 weeks of nicotine treatment. The percentages are calculated as UPE decline in nicotine treated animals relative to the non nicotine treated control group. Although no impressive differences were achieved, nicotine treatment significantly mitigated rise in UPE by about 10%, 20% and 16% in the N20, N60 and N100 groups resp.

Figures 1A and 1B



Glomerular histomorphological analysis

PAS staining was done to quantify GS. MWF rats developed de novo GS over time. At the end of the study, CON had higher GS than nicotine treated groups ($p=0.001$). Long term nicotine treatment led to a significant, approximately 50% reduction in GS score in N60 and N100 vs CON (both $p=0.02$) (figure 2). Similarly podocyte loss and desmin positivity were higher in CON vs baseline ($p= 0.04$ and $p=0.01$ resp.). Long term nicotine treatment led to preserved podocin staining and lower desmin deposition (figure 3 and figure 4 resp.).

Figure 2 (FGS)

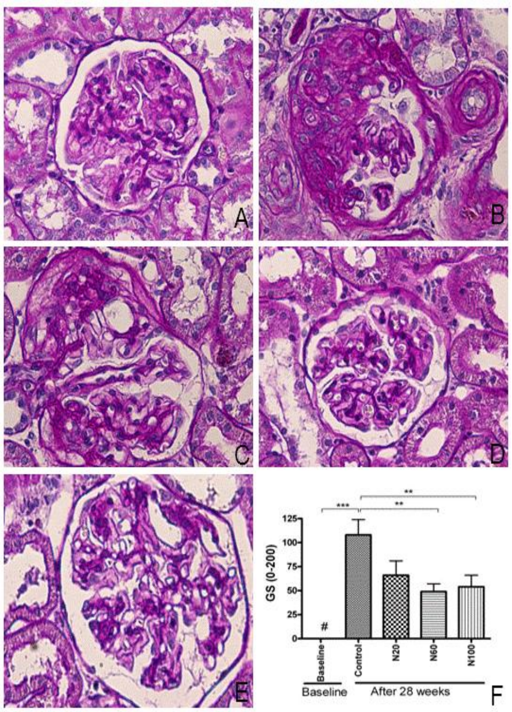


Figure 3 (Podocin)

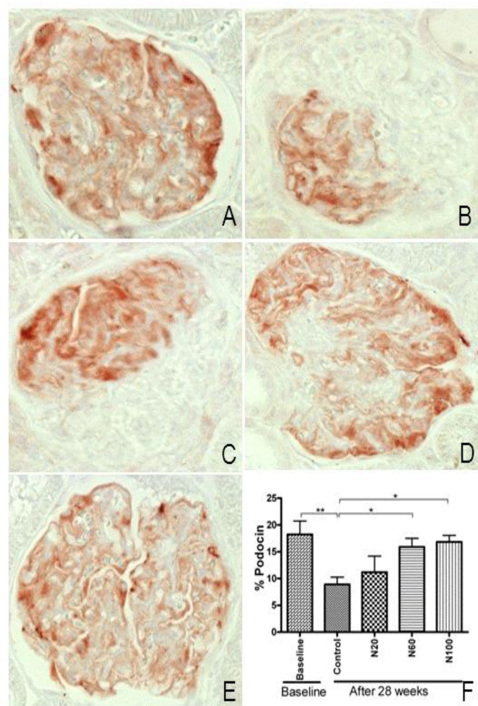
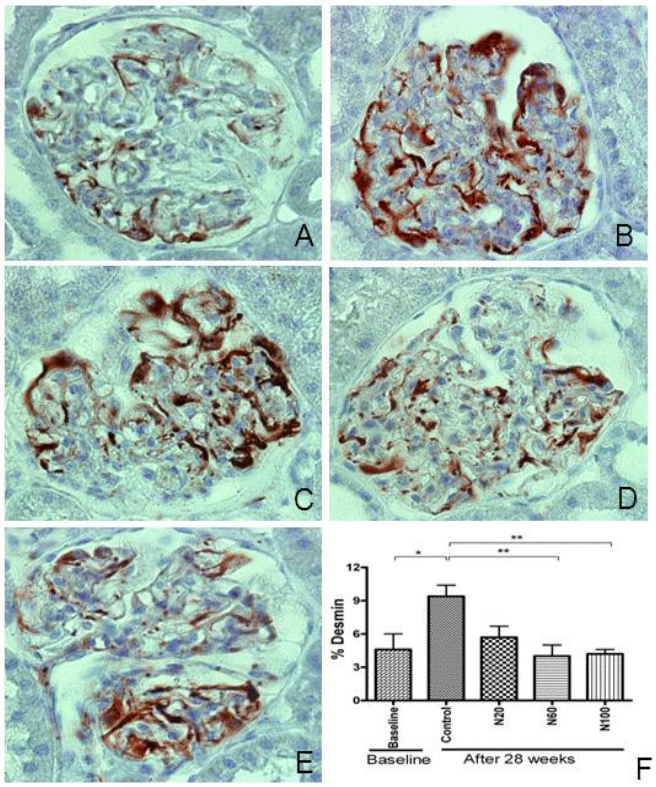


Figure 4 (Desmin)



Long term nicotine treatment led to a significant, approximately 50% reduction in Desmin score in N60 and N100 vs CON (both p=0.01) (figure 4).

Renal infiltration of monocyte/macrophages and myofibroblasts

Nicotine treatment in N60 and N100 significantly reduced the amount of ED1 positive macrophages in the kidney when compared to CON (p=0.001) (figure 5). Myofibroblasts were stained with α-SMA and represent a major cell type involved in interstitial matrix deposition. Figure 6 shows that N60 (p=0.01) and N100 (p<0.05) had significantly reduced α-SMA positivity in the kidneys when compared to CON.

Figure 5 (Macrophages/monocytes)

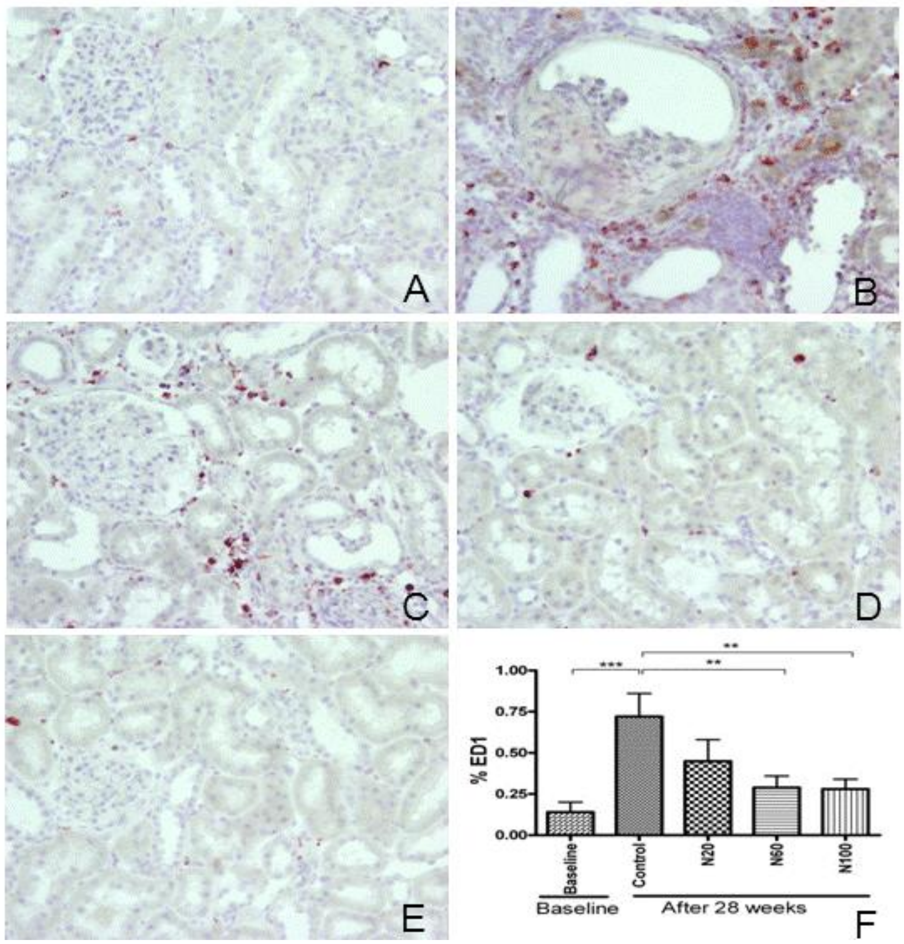
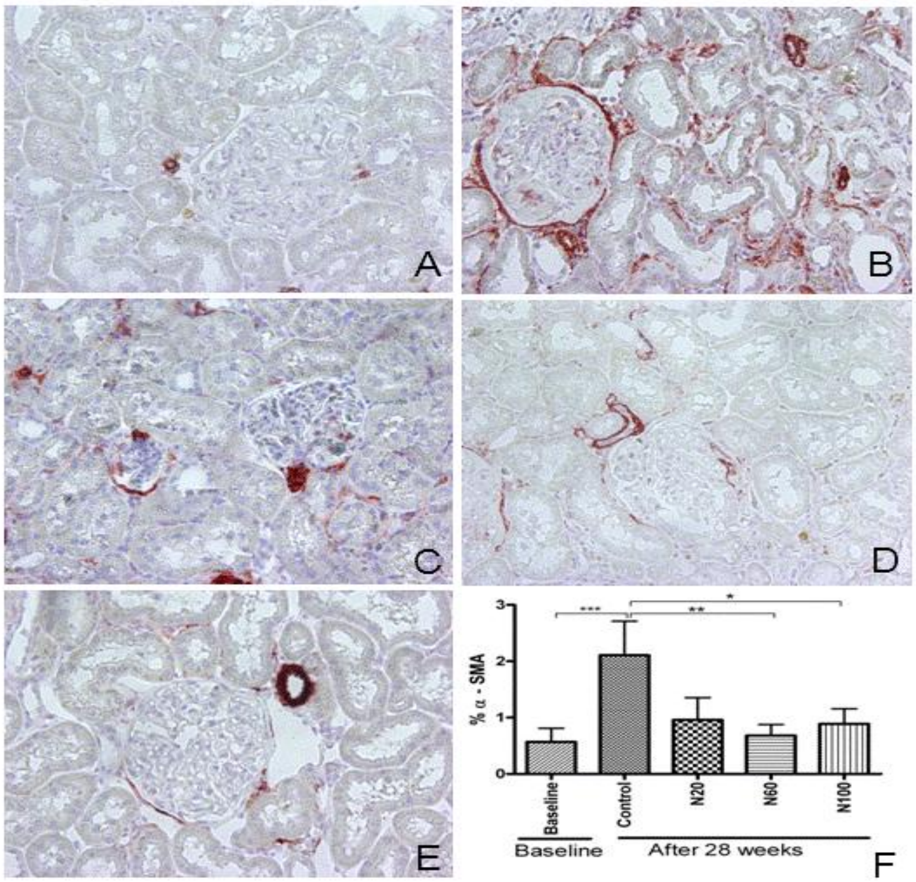
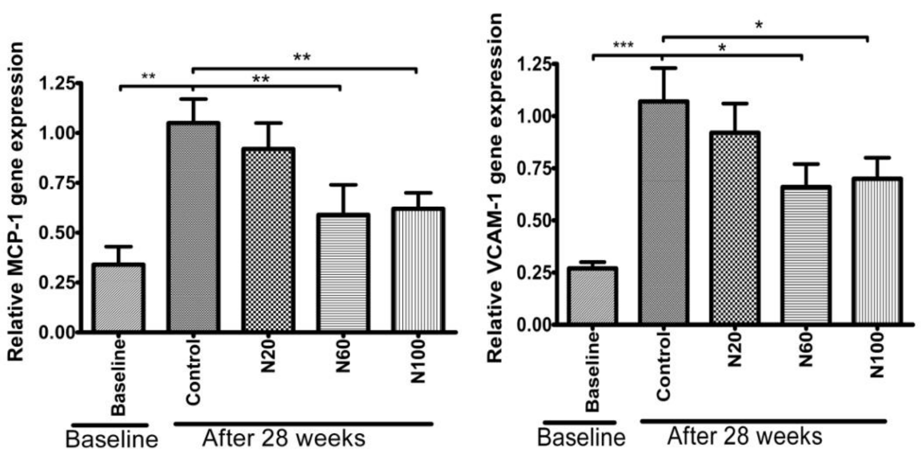


Figure 6 (α-SMA staining)



qPCR analysis

Since the influx of ED-1 positive monocytes and macrophages was reduced upon oral nicotine, we evaluated by qPCR renal expression of MCP-1 and VCAM-1, the former being a major chemo-attractant and the latter being an important adhesion molecule for monocytes and macrophages. Both, the expression of MCP-1 and VCAM-1 were higher in CON at the end of the study compared to baseline (both p<0.01, Figure 7A and 7B resp.). Nicotine treatment dose-dependently lowered the expression of total MCP-1 in N20, N60 and N100. However N60 (p<0.01 vs CON) and N100 (p<0.01 vs CON) reached statistically significance Moreover, nicotine treatment lowered the expression of total VCAM-1 as well in N60 (p<0.05 vs CON) and N100 (p<0.05 vs CON).



Discussion

Our results demonstrate that long term oral nicotine improves renal function and morphology and reduces renal inflammation in the Munich-Wistar-Fromter rat. As anticipated in this model, the CON group developed loss of kidney function, GS and tubulo-interstitial inflammation. The glomerular as well as interstitial damage was reduced by oral nicotine in a dose dependent manner. Improvement was also seen at mRNA level by down regulation of the inflammatory markers MCP-1 and VACM-1.

Nicotine and cigarette smoking have been shown to elicit an acute systemic adrenergic response, resulting in increases in blood pressure and heart rate in both healthy subjects and renal patients (37). It is, however, questionable whether these effects are sustained on the longer-term. In an animal study, the same group found no effects on blood pressure of long-term administration of nicotine in combination with cigarette smoke extract (38). Consistent with these findings, we did not find any statistical significant change in blood pressure in our long-term animal study. Whereas our assessment of blood pressure by tail-cuff is not gold standard, it seems implausible that an effect on blood pressure would go unnoticed altogether.

On one hand, nicotine is known to induce endothelial nitric oxide synthase (eNOS) which is vasodilatory in isolated perfused kidney (39) while on the other hand, it is known that that acute or chronic nicotine attenuates the renal vasodilatory responsiveness to some vasodilator pathways (40). Therefore, a direct effect on glomerular pressure either by efferent vasodilatation or afferent vasoconstriction can not be excluded, in particular because UPE was slightly higher in CON groups than in nicotine treated groups. The lowering of UPE was persistent throughout the experiment. This lowering in UPE might have contributed to the observed renoprotective effects of oral nicotine.

It is known that nicotinic receptors are present on macrophages, peritubular capillaries, dendritic cells and vascular smooth muscle cells (21,27,41). Previous studies have shown that nicotine reduces monocyte/macrophage influx in tissues via the α-7NAchR pathway and reduces endothelial activation (30,42). Nicotinic agonists attenuate the activation of macrophages (43). We also showed that macrophage infiltration was reduced in nicotine treated animals. Various cytokines, including IFN-γ, IL-6 and lymphocyte activation, are implicated in the activation of macrophages (44,45). Nicotine is known to down regulate these inflammatory cytokines and this effect could play a role in reduction of glomerulosclerosis and interstitial fibrosis (29,46,47). We observed reduced glomerulosclerosis and lower density of myofibroblasts. These observations indicate an anti-fibrotic effect of nicotine. However, we cannot discriminate between a direct nicotinic anti-inflammatory effect and a secondary nicotinic effect via reduction in UPE. We investigated the therapeutic effects of oral nicotine in 3 different doses. There was clear reduction of GS, UPE, podocytopathy, desmin deposition, ED-1 influx and α -SMA accumulation at N20 which is in between CON and N60. For 60 and 100 except for α-SMA, the effects were more or less similar, suggesting that there is saturation of nicotinic receptors at and beyond 60 mg/l.

Few studies have investigated the role of nicotine in rodent models of kidney disease. Although findings of our study are consistent with many studies, some studies even reported opposite results. On one hand, our findings are in line with findings of Yeboah et al and Sadis et al, where they found anti-inflammatory effects of oral nicotine in rat models of I/R induced kidney damage (25,48). On the other hand, a deteriorating effect of nicotine was found in mice models for diabetes (49) and ischemia reperfusion injury (50). How might these differences been explained? First of all, it is always cumbersome to compare rodent models with respect to strain differences (mouse vs rat) and source of delivery, sex (male vs female), age, and dosage scheme of the drug. More importantly is to realize that effects of nicotine are evaluated in different models of renal disease. Our model, the MWF rat, is a proteinuric model (51). That means that proteinuria is the driving force behind renal damage. Proteinuria can be reduced by reducing glomerular pressure. Most likely, nicotine reduced glomerular pressure and/or reduced proteinuria, and consequently renal function and morphology and renal inflammation is reduced. The studies that reported negative effects of oral nicotine used diabetic mice and mice that underwent ischemia/reperfusion (45, 46). Driving forces behind renal damage in these models are hyperglycemia and lack of oxygen respectively, which apparently uses other activation and signalling routes that are less sensitive to interference with nicotine. Moreover, the MWF rat model used by us demonstrates more chronic inflammation compared to the db/db mice and the chronic phase of ischemia/reperfusion. This might explain why anti-inflammatory actions of nicotine were more clear in our model.

Smoking is a risk factor for cardio-vascular diseases, kidney disease and lung cancer (13,52).

Nicotine is one of the most addictive components of cigarette smoke. Our study indicates that oral nicotine besides reducing direct harmful effects from cigarette smoking and overcoming the addiction of smoking, reduces inflammation in kidneys. However nicotine binds to all nicotinic receptors and could lead to systemic side effects. Binding of nicotine to neural nicotinic receptors makes it addictive in nature. Caution should be taken in prescribing nicotine due to its potential cardiovascular side-effects. Further research is necessary to assess whether nicotine without concomitant exposure to other constituents normally present in cigarette smoke or carbon monoxide is harmful from a cardiovascular perspective (53,54). Thus, other specific α-7nAchR agonists could be of therapeutic use, particularly if these compounds would be devoid of addiction effects.

To the best of our knowledge studies with long term nicotine are lacking despite nicotine being used chronically in clinical settings. Therefore we investigated nicotine usage in a long run and found that nicotine is reno-protective in MWF rat model of spontaneous proteinuria. It should, however, be realized that these renoprotective effects may be limited to the model we used. MWF rats have spontaneous progressive proteinuria and glomerular podocytopathy (51,55). Therefore, it can not be excluded that similar beneficial effects are not present in other models of progressive proteinuria and kidney disease. This deserves additional experiments. The kidney function was measured with creatinine clearance so as to avoid any effects of change in muscle mass (56). We found that nicotine treatment improved creatinine clearance. However kidney function as measured by creatinine clearance is not the gold standard specially in rodents since there is a considerable amount of tubular secretion of creatinine. Measuring renal function with exogenous renal markers, such as inulin, iothalamate or iohexol, although considered to be the gold standard, is time consuming, invasive and cumbersome (57). We therefore cannot exclude the nicotine induced tubular secretion of creatinine in the nicotine treated animals. Our conclusion of renoprotective effects of nicotine however are based not solely on the improvement of creatinine clearance but also on the concomitant moderate attenuation of UPE and renal morphological improvements. Furthermore, although we show that oral nicotine results in lowering of proteinuria and renal inflammation, the exact mechanism responsible for renoprotection is not identified by our study and deserves further research, e.g. by further studies with renal denervation.

In conclusion, our data show that long term oral nicotine improves kidney function, reduces proteinuria, renal inflammation and glomerulosclerosis in Munich-Wistar-Fromter rats. This novel action of nicotine could be evaluated as a potential additional therapeutic option for treating proteinuric and/or inflammatory kidney diseases.

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Chapter 6

Nicotine Modulates Neointima Formation in Intra Renal Arteries

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Chapter 6

Abstract

Aims: Transplant vasculopathy (TV) is an important component of chronic allograft rejection. TV is associated with neointima formation. Inflammation plays a central role in the process of neointima formation. Nicotine is known to be anti-inflammatory in nature. We recently found indication that nicotine prevents neointima formation and aimed to investigate whether it could prevent neointima formation in another model of neointima formation.

In a first experiment, we found neointima formation in intrarenal arteries in the hypertensive Munich-Wistar-Frömter rat model of spontaneous proteinuria. In this model, nicotine treatment with 60 mg/l and 100 mg/l drinking water reduced neointima formation by 90 % and 85 % respectively compared to non-nicotine treated rats (both $p<0.01$ vs non-nicotine treated control rats). In a subsequent experiment, we aimed to evaluate whether nicotine treatment could prevent formation of TV in a rat renal allo-transplantation model. Similar to the findings of the first experiment, nicotine treatment (60 mg/l) reduced neointima formation by 79% compared to non-nicotine treated rats ($p=0.055$). Other physiological parameters like blood pressure and body weight remained comparable among groups in both the experiments.

This study reports presence of neointima formation in intra renal arteries of a MWF rat model of spontaneous kidney disease and further demonstrates that nicotine reduces neointima in this model. Furthermore, nicotine tended to prevent neointima formation in a model of TV. Therefore we suggest to further investigate nicotine as an additional therapy for treatment of neointima formation which is commonly seen with transplant vasculopathy, to delay progressive renal function decline and long term graft failure.

Introduction

Transplant vasculopathy (TV) is an important component of chronic allotransplant rejection. TV is a complex process with poorly understood pathophysiology. A prominent histological feature of TV is neointima formation resulting from infiltration of inflammatory cells, proliferation of smooth muscle cells and accumulation of extracellular matrix (1,2). The end result is progressive luminal occlusion and end organ failure. Therefore long term graft survival can be improved by reducing neointima formation associated with TV. Various mechanisms have been proposed to understand the origin of neointima formation including changes in hemodynamics, disruption of endothelial barrier and ischemia reperfusion injury (3-6). Inflammation is, however widely argued to be a central event in the process of neointima formation, triggered by vascular injury and maintained through autocrine or paracrine mediators (7).

Inflammation can be modulated via non-neuronal cholinergic pathway (8,9). The effects are thought to be mediated via nicotinic acetylcholine receptors (nAchRs), mainly α7-nAchR. Non neuronal α7-nAchR has been described to be present on macrophages as well as on endothelial cells (ECs) (10). Nicotine is non-specific agonist of nAchRs. The binding of nicotine to α7-nAchR is known to be anti-inflammatory (11,12). It has been shown that activation of nAchRs ameliorates diseases of inflammatory origin like ulcerative colitis and sepsis and ameliorates endothelial dysfunction (13-15). It has also been shown that nAchRs are down regulated in inflammatory conditions and nicotine pretreatment up regulate the nicotinic receptors (16). In a Munich-Wistar-Frömter (MWF) rat model of spontaneously proteinuria, we have recently shown that long term nicotine ameliorates decline in kidney function and progressive renal structural damage (17). In this model, we observed neointima formation in intra-renal arteries and partial prevention of this formation in animals treated with nicotine. We hypothesized that nicotine treatment could also slow down the development of neointima formation in association with TV.

The aims of this study are following: 1) to report presence of neointima formation in intra renal vessels of a MWF rat model of spontaneous kidney disease and effects of nicotine on the neointima formation. 2) To investigate effects of nicotine on neointima formation in a rat renal transplantation model of TV.

Material and Methods

Animals

24-wk old Munich-Wistar-Fromter rats were obtained from Harlan (USA), 10 wk male Wistar Furth rats were obtained from Harlan (Zeist, The Netherlands) and 10 wk female Dark Agouti rats were obtained from Charles River (Maastricht, The Netherlands). All animals were housed in temperature controlled 12h light/12h dark rooms and had free access to food and drinking water. All animals received human care in compliance with the Principles of Laboratory Animal Care (NIH Publication no. 85-23, revised 1996) and the protocols of both experiments were approved by Animal Ethical Committee of University of Groningen.

Experiment 1: Spontaneously proteinuric MWF rat model

Spontaneously proteinuric MWF rats (n=44) were used. Division of rats in different treatment groups have been previously described (17). Briefly for baseline renal vascular measurements MWF rats (n=6; Baseline) were sacrificed at week 24 of age. The other animals were randomly assigned to four different groups. Rats were given either of 3 different concentrations 20 mg/l (n=9; MWF20), 60 mg/l (n=9; MWF60) and 100 mg/l (n=10; MWF100) of nicotine mixed with 0.5% sodium saccharin as sweetening agent ad-libitum in drinking water. Rats in the control group (n=10; CON) only received water sweetened with 0.5% sodium saccharin. Animals were sacrificed after 28 weeks of treatment (i.e. at 52 weeks of age).

Experiment 2: Kidney allo-transplantation model

Left orthotopic kidney transplantation was carried out from female Dark Agouti (DA) rats to male Wistar Furth (WF) rats as previously described (18). Briefly kidneys from donor DA rats were flushed and stored in cold saline. Cold ischemia time ranged from 12-20 minutes. In recipient WF rats, renal vessels were clamped and the left kidney was removed. During surgery renal vein, artery and ureter were end to end anastomosed and vascular clamps were removed. Warm ischemia time of 25 min was kept constant during all the transplantations. Rats received standard analgesic, buprenofine (Temgesic) 0.01 mg/kg, twice on the day of transplantation and once on the second day of transplantation. To prevent acute rejection recipients received cyclosporine A (5 mg/kg/day; Sandimmune, Sandoz Pharma AG, Basel, Switzerland) subcutaneously for first 10 days post transplantation. The contralateral native (right) kidney of WF rats was removed at day 14, and the rats were followed till the end of experiment.

Nicotine treated groups received either 30 mg/l (WF30; n= 10) or 60 mg/l (WF60; n= 10) of nicotine in drinking water ad libium throughout the experiment. Recipients received nicotine starting 2 days prior to transplantation to get acclimatized to nicotine. Control group (CNT) received no nicotine (n=10). Animals were sacrificed at 9±2 weeks (mean 63 days) after transplantation.

Experimental procedures during the experiments and laboratory measurements

Body weight was measured weekly. Blood pressure was measured by tail cuff method after appropriate training of the rats. Venous blood was withdrawn under isoflurane anesthesia in heparin-containing tubes and plasma was stored in -80 °C until analysis. 24-h urine was collected in metabolic cages and stored at – 20 °C until analysis. 24-h water and food intake were measured in the metabolic cages. Animals were sacrificed under isoflurane-induced general anesthesia by cutting the diaphragm and cardiac bleeding. Animals were

flushed with 0.9% NaCl, kidneys were harvested and partly stored in -80 °C and partly fixed in 4% formalin followed by embedding in paraffin.
Plasma creatinine and urinary protein were measured by multi-test analyzer system (Roche Modular; F. Hoffmann-La Roche Ltd, Basel, Switzerland). 24-h urinary protein excretion was calculated from urinary protein concentration and 24-h urinary output.

Morphometric analysis of renal vessels
4 μm thick formalin fixed paraffin sections were deparaffinized and were stained with Verhoeff's Masson staining. With this staining the internal elastic lamina (IEL) and external elastic lamina (EEL) were stained in black and the interstitial tissue was stained in red.
In every rat, in a blinded fashion, all the identifiable elastin positive intra renal vessels were evaluated for luminal diameter, neointima area and medial area using Olympus BX50 research microscope and Cell^B Olympus programme (Olympus Europe, Hamburg, Germany). Diameter of vessels calculated as the mean length of two straight lines drawn from IEL and passing through the center of vessel. The areas enclosed by lumen, IEL and EEL were measured. The area between the lumen and IEL was described as neointimal (NI) area and the area enclosed between IEL and EEL was described as medial area. The ratio between NI area and medial area was calculated. [NI area (if present)/ area enclosed by IEL] X 100 is described as percentage of luminal occlusion.

Statistical analyses
Statistical analysis was performed using Spss 14. Figures were made using GraphPad Prism (version 4.00; GraphPad Software, Inc., San Diego, CA). Differences among the groups were analyzed using ANOVA with Tukey post hoc. Differences between start of the experiment and end of the experiment were analyzed using independent samples t-test. Log rank test was used to test survival differences among groups. $P < 0.05$ was considered statistically significant.

Results

Experiment 1

Survival and other physiological parameters of animals

One animal in N60 was sacrificed prematurely because of tooth malformation due to which the animal was unable to eat and lost weight. One animal in N20 was sacrificed because of a bone tumor. The tumor formation is unlikely to be related to nicotine since it was a sporadic case which might be related to aging. Both the animals were excluded from all analyses.

The physiological parameters are summarize in table 1.

Table 1

Clinical parameters of spontaneous proteinuric MWF rat model and renal allo-transplantation model.

	Spontaneously proteinuric MWF model					Renal allo-transplantation model		
	Baseline	Control	MWF20	MWF60	MWF100	CNT	WF30	WF60
Body weight (g)								
Start of Experiment	323 (5)	347 (8)	334 (5)	334 (6)	351 (5)	286 (16)	278 (20)	279 (19)
End of Experiment	_	385# (10)	366# (10)	367# (8)	375# (7)	322# (29)	314# (25)	308# (4)
Water intake (ml/24h)								
Start of Experiment	28 (5)	21 (2)	21 (3)	19 (3)	18 (4)	15 (4)	13 (6)	14 (6)
End of Experiment	_	11# (6)	10# (6)	16 (10)	10# (3)	27# (4)	31# (9)	18 (9)
Urine output (ml/24h)								
Start of Experiment	25 (4)	17 (1)	16 (2)	19 (3)	18 (3)	14 (4)	12 (4)	12 (2)
End of Experiment	_	15 (6)	12 (6)	10# (4)	10# (3)	31# (3)	30# (6)	24# (5)
SBP (mmHg)								
Start of Experiment	179 (8)	174 (5)	174 (4)	180 (3)	181 (7)	150 (24)	150 (13)	169 (18)
End of Experiment	_	181 (6)	180 (6)	176 (5)	174 (5)	183# (26)	194# (8)	191# (17)
Plasma creatinine (µmol/l)								
Start of Experiment	26 (4)	22 (2)	20 (3)	21 (2)	21 (2)	20 (4)	21 (3)	17 (1)
End of Experiment	_	50# (7)	37# (5)	31#* (1)	32#* (2)	102# (41)	80 (30)#	73# (25)
UPE (mg/24h)								
Start of Experiment	47 (24)	42 (8)	48 (7)	36 (5)	41 (5)	10 (2)	8 (5)	10 (3)
End of Experiment	_	136# (8)	122# (6)	109#* (7)	110#* (11)	73# (41)	122# (780	103# (63)

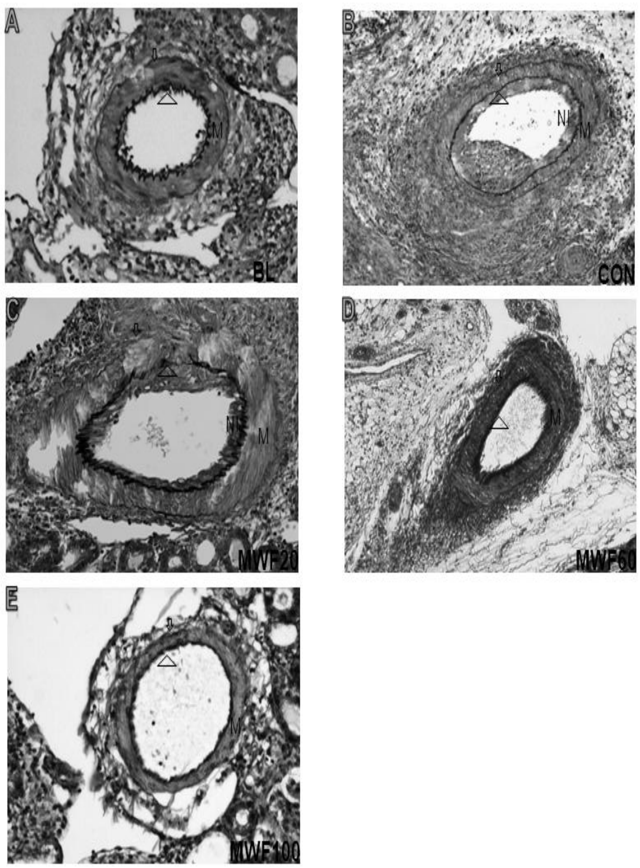
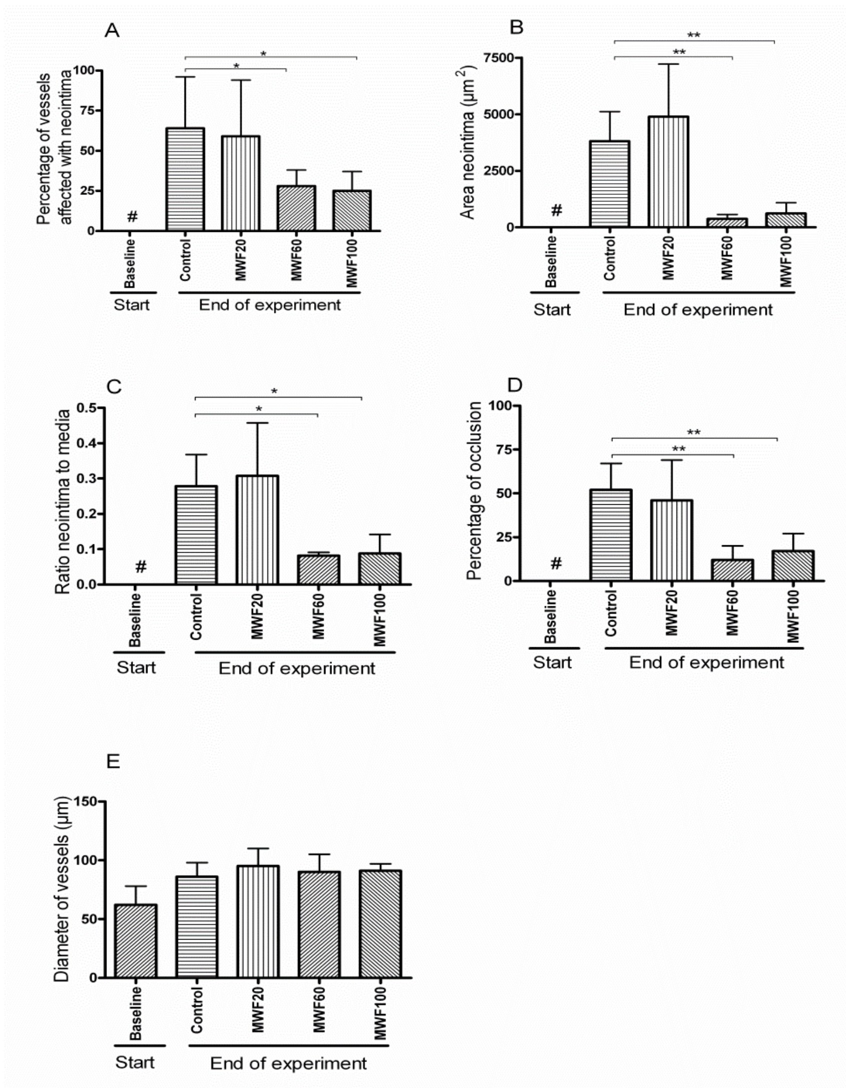


Figure 1: (Representative photos of vascular lesions) Representing intral renal artery from each group is depicted in figure 1. At the age of 24 weeks none of intra renal arteries in MWF rats had presence of neointima. In the animals at 52 weeks of age, significant neointima formation was present in rats not treated with nicotine. The percentage of vessels affected with neointima was reduced in nicotine treated animals compared to non-nicotine treated animals (figure 2A). Results for presence of neointima (figure 2B), neointima to media ratio (figure 2C) and luminal occlusion (figure 2D) were consistent. Renal diameter was comparable among the non-treated control animals and the nicotine treated animals (figure 2E).

Figure 2 (quantification of vascular lesions of long term nicotine experiment)

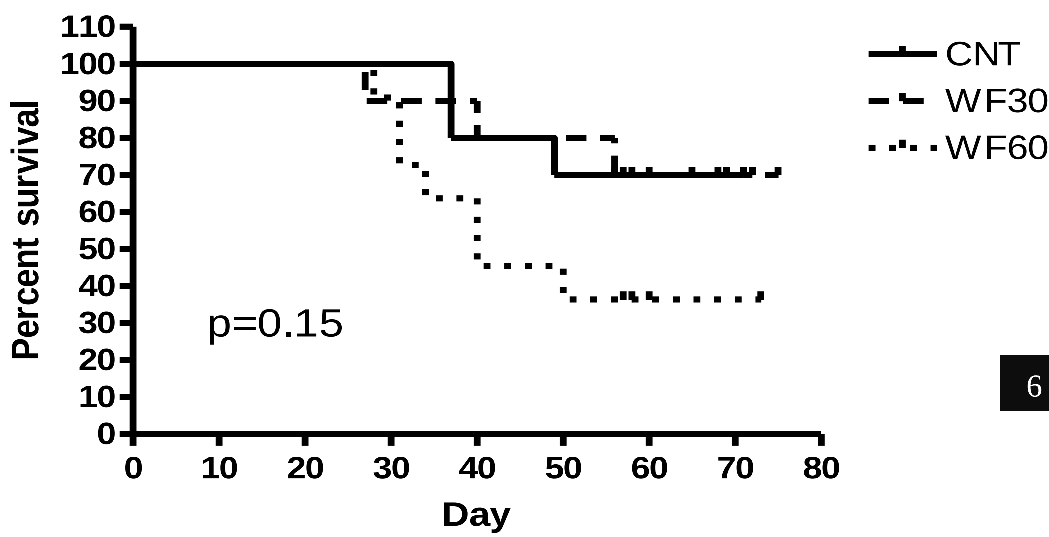


Experiment 2

Survival and other physiological parameters of animals

This experiment included 30 male WF recipients. During follow-up from 2-9 weeks, pre-term graft failure occurred in 3 rats (30%) in CNT group , in 4 rats (40%) in WF30 group, and in 6 rats (60%) in WF60 group. The survival of animals in different groups is depicted by Kaplan-Meier curves in figure 3. The rats that were sacrificed before the end of the experiment (before 9 weeks after transplantation) were excluded from all analyses. Renal parameters (serum creatinine and creatinine clearance) of the excluded animals were comparable among the groups (data not shown). Accordingly, the analyses were performed with 17 rats i.e. CNT (n=7), WF30 (n=6) and WF60 (n=4).
The physiological parameters are summarize in table 1.

Figure 3



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Morphometric analysis of renal vessels

Representing intra renal artery from each group is depicted in figure 4. The percentage of vessels with neointima formation was reduced in 60 mg/l nicotine treated animals compared to non-nicotine treated animals (figure 5A). The reduction of neointima in 60 mg/l nicotine group was borderline significant when compared to control group (p=0.055) (figure 5B). There was trend towards reduction of the neointima to media ratio in 60 mg/l nicotine groups when compared to non-nicotine treated control group (figure 5C). The ratio between neointimal area and total lumen area represents the percentage of occlusion. The nicotine treatment with 60 mg/l and reduced the luminal occlusion compared to non-treated group by 50% (figure 5D). The renal diameter was comparable among the non-treated control animals and the nicotine treated animals (figure 5E).

Figure 4 (Representative photos of vascular lesions)

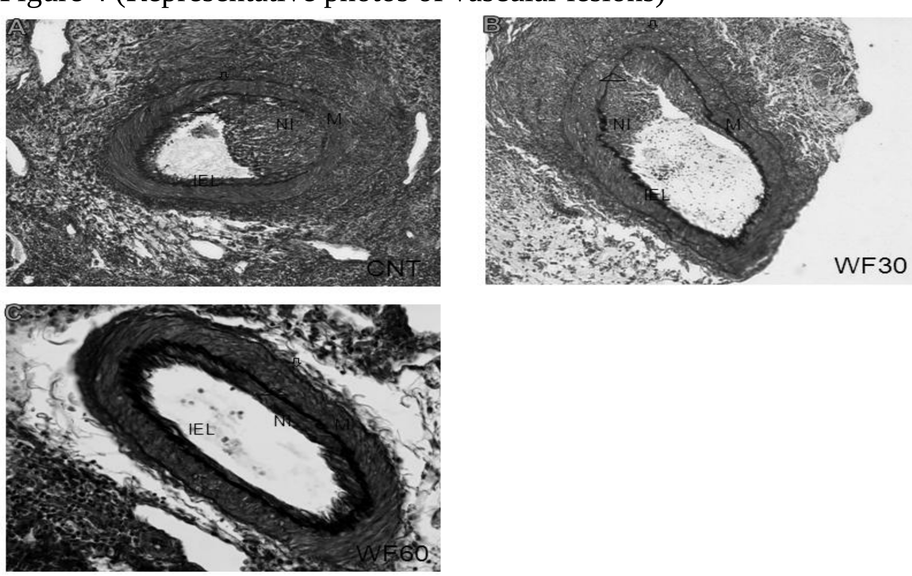
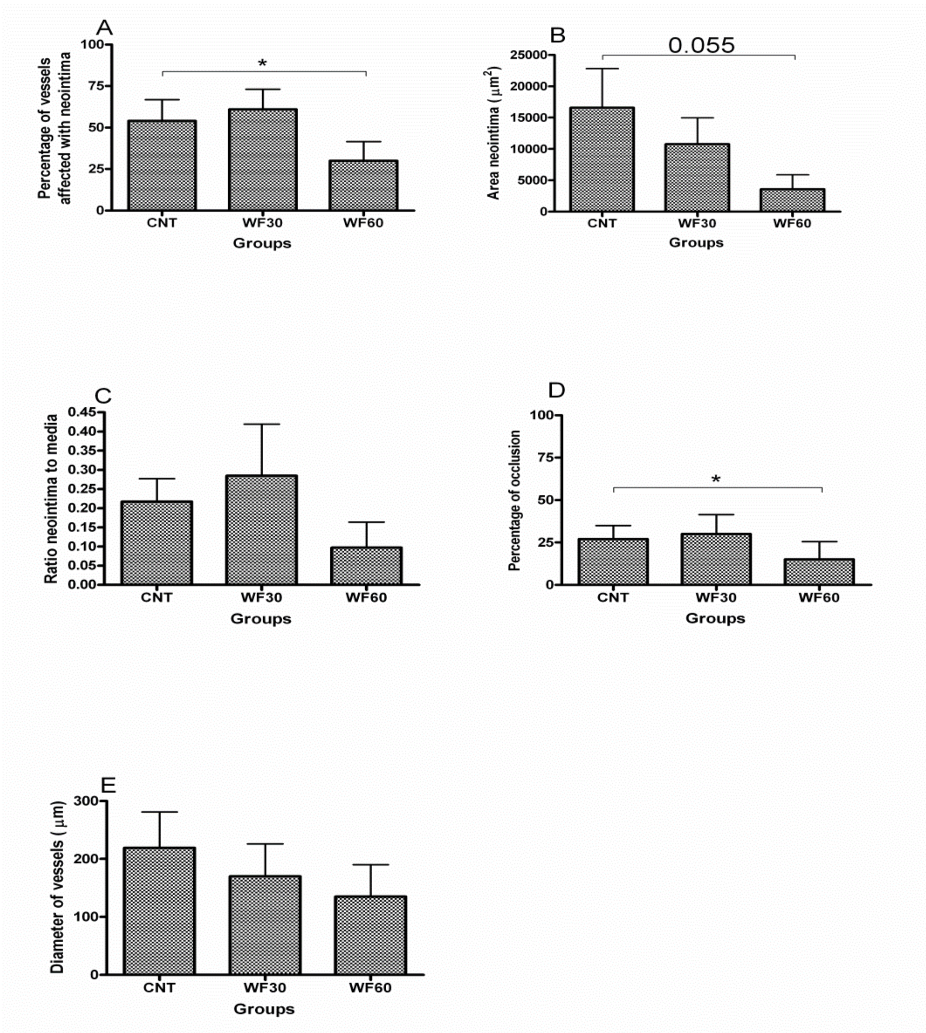


Figure 5 (quantification of vascular lesions of transplantation experiment)



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Chapter 6

Discussion

In this study we describe that nicotine treatment reduces neointima formation in intra renal arteries in two different rat models renal disease with neointima formation. The specific effect of nicotine on intra renal artery neointima formation is independent of change in blood pressure.

Transplant vasculopathy is an important component of chronic transplant dysfunction. The pathophysiology of neointimal lesions is unclear, therefore poses great difficulties in designing appropriate therapy. However endothelial cell activation and inflammatory process play a crucial role in pathways leading to progression of neointima formation (19). Therefore therapies targeting those routes might prove to be promising.

In a rat model of spontaneously proteinuria (experiment 1) we observed the presence of neointima in intr-renal arteries. The presence of neointima formation in the MWF rat model has not been described previously. In this study we therefore report the finding. Additionally we found that neointima formation was ameliorated by nicotine treatment where higher dose nicotine treated animals had lesser formation of neointima. It was therefore suggestive that nicotine is arterio-protective.

This finding led us to investigate nicotine effects on a rat model of TV in the setting of kidney transplantation. In this experiment investigation with 2 different concentrations of nicotine (30 mg/l and 60 mg/l) were carried. The concentrations were chosen based on observation from experiment 1 that nicotine effects were comparable at concentrations 60 mg/l and 100 mg/l. Neointima formation in kidney transplantation contribute to chronic transplant dysfunction (CTD), thus therapies aiming at reducing transplant vasculopathy are clinically relevant. We thus investigated nicotine effects in an orthotopic allo-transplantation model from female Dark Agouti into male Wistar Furth rats (18). The allo-transplanted animals developed renal function decline along with systemic hypertension. The transplanted kidney showed signs of CTD like glomerulosclerosis, interstitial fibrosis, tubular atrophy, and transplant vasculopathy with neointima formation. Neointima formation was characterized by concentric intimal hyperplasia with luminal occlusion. Nicotine treatment in the highest dose showed a clear tendency towards reduction in amount of neointima. We however did not find any reduction in proteinuria, however a tendency of improvement in kidney function was observed, which did not reach statistical significance, probably related to a small number of animals in nicotine treated groups resulting in a statistical power problem.

Nicotine effect on vessels has been previously studied. Rodella et al used nicotine treatment as model for neointima formation while aiming to investigate anti-oxidant properties of melatonin (20). They found that nicotine treatment for 56 days induced neointima formation in aorta. Vazquez-Padron et al showed that VSMC proliferation plays an important role in neointima formation in injured right Iliac-artery of rats pretreated with nicotine (21). In the study by Vazquez-Padron et al the nicotine treated rats developed hypotension which is in contrast to our model. Hypotension related hypoxia might be an inducible factor for nicotine associated effect of neointima formation, although we did not find effects of nicotine on blood pressure. Hamasaki et al concluded that administration of nicotine tend to accelerate the intimal hyperplasia upon removal of the endothelial lining (22). At present we cannot explain why neointima formation in aforementioned studies is increased by nicotine, while we find protective effects in two independent rat renal models of kidney disease. It might be that the smaller renal arteries (ranging from 50-250 um) responds differently from larger arteries of the body. Besides to that, both renal models are characterized by increased blood pressure, which might influence nicotine effects. Additionally dose of nicotine, duration of treatment or induction of neointima by mechanical or non-mechanical injury might be associated with the different findings. Though we did not aim to study the mechanism of action of nicotine effects on neointima formation but anti-inflammatory action of nicotine might well play a role. Nicotine is known to be anti-inflammatory acting mainly via α7nAchRs (23,24). α7nAchRs are present on monocytes and vascular endothelium, and also on vascular smooth muscle cells and fibroblasts (8,25,26). Agonists of nicotinic receptors inhibits activation of monocytes and endothelial cells. We have also shown lower influx of monocytes/macrophages in interstitium of nicotine treated MWF rats (17). Therefore it might be that nicotine inhibited monocyte activation leading to slower progression in neointima formation. However this requires further investigation with nicotinic receptor knock out models.

Another key player for nicotine related effects might be nitric oxide (NO). Vascular integrity is necessary to maintain normal functioning of blood vessels. Under healthy conditions the integrity is maintained by secreted NO from intact endothelium (27). The endothelium lining gets damaged in transplantation setting and under conditions of high blood pressure. It has been shown that supplementation with exogenous NO can repair the damaged vascular integrity (28,29). nAchRs are present on endothelium and nicotinic agonists are known to induce eNOS production and secretion and increase formation and secretion of NO from endothelial cells. NO causes vasodilatation, decreases SMC activation and proliferation thus inhibit progression of neointima formation (30). nAchRs are also shown to present on macrophages. Agonists of such receptors lead to inhibition of monocytes activation, lesser chemo-attractant secretion which is required to sustain inflammation and reduced proliferation of VSMCs. Moreover local chemoattractant properties of macrophages may be reduced by nicotine. Recent studies with drug eluting stents which deliver NO also showed to be beneficial in preventing in stent restenosis after traumatic injury, are line with our finding (3).

In this study we describe that nicotine reduces progressive neointima formation in two different rat models of renal neointima formation. However there are few limitations of the study. Like in all animal studies the results of the study should be interpreted with caution when relating to human pathology. Because of the full HLA mismatch between the donor and recipient in experiment 2 there was severe damage to the transplanted kidney, resulting in a high number of dropouts in all the groups. Although the drop-out rate was not significantly different among the groups, most rats dropped out in the highest (60 mg/l) nicotine group. However in experiment 1 the same dose (60 mg/) and a higher dose (100 mg/l) were well tolerated. Besides to that, in experiment 2 we cannot exclude a certain selection bias in the surviving animals. Lastly, the resulting low number of animals that were included in the analyses of experiment 2 raised a power problem for proper statistics.

In summary, we describe that nicotine treatment reduce the amount of renal neointima formation in two different rat models of renal neointima formation. We suggest to further investigate nicotine as an additional therapy for treatment of neointima formation, to delay progressive decline of renal function and chronic transplant dysfunction in renal allo-transplantation.

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Chapter 7

General Discussion and Future Perspective

Chapter 7

Summary and general discussion

The studies presented in this thesis aimed to explore the effects of smoking and nicotine on the kidney. Diabetes, hypertension and obesity are well established risk factors for cardiovascular and renal diseases (1-6). Association between life style related risk factors and kidney diseases is now also being recognized. Potential roles of salt intake, physical activity, sleep, and psychosocial stress on the progression of chronic kidney disease (CKD) are increasingly being evaluated (7-12). Despite the development of newer therapeutic modalities and improved patient management, many patients still progress to End Stage Renal Disease (ESRD) which necessitates renal replacement therapy (13-15). Therefore, it is important to identify new risk factors, specially those which are modifiable, to develop new intervention strategies. Smoking is a modifiable risk factor associated with cardiovascular diseases and with progression of diverse kidney diseases, like diabetic nephropathy, IgA nephropathy, hypertensive kidney disease and so forth. In the same line of research, in this thesis we investigated effects of smoking on native and transplanted kidneys.

Smoking and deterioration of kidney function

Smoking has recently been identified as a risk factor for development and progression of chronic kidney disease in native kidneys (16-18). Cigarette smoking can cause renal damage by causing renal vasoconstriction, thus eliciting a decline in effective renal plasma flow, by toxic effects from oxidative components and by exposure to heavy metals (19-25). Nicotine is one of the main components of cigarette smoke. Nicotine is also thought to be injurious to the kidneys (26-29). One of the contemporary theories is that detrimental effects of smoking on kidneys is through the action of nicotine on the sympathetic nervous system, thereby inducing intrarenal vasoconstriction and parenchymal ischemia (22,30,31). It should be noted that the kidneys of renal transplant recipients receive no innervation. If kidneys of renal transplant recipients would be susceptible to detrimental effects of smoking, it would be an indication that mechanisms other than an effect of nicotine on the sympathetic nervous system are involved. In **chapter 2** of this thesis we therefore set out to investigate the association of cigarette smoking on decline in kidney function and development of graft failure in renal transplant recipients (RTR). We found that in current smokers the decline in kidney function was faster than that in past and never smokers, with similar slower rates of decline for past and never smokers. In line with this, we found that current smokers had a higher risk for development of graft failure than non-smokers or ever smokers. If nicotine related sympathetic activation was a cause of the nephrotoxic effects of smoking, such an effect would not have been observed in the RTR model of renal denervation. Therefore it is suggestive that mechanisms other than nicotine induced sympathetic hyper-activation are involved.

As smoking is a main disease progression factor in chronic pulmonary disorders, many patients that require a lung transplant have a history of heavy smoking. Approximately 60% of lung transplant recipients have a history of smoking (32). However, they have to stop smoking before being considered for transplantation. In most lung transplant recipients renal function deteriorates progressively after transplantation, often resulting in CKD (33), with a likely role for CNI toxicity, superimposed on the frequently occurring bout of hypotension-elicited AKI in the early post-operative phase. Therefore CKD is being recognized as a serious complication after lung transplantation (34). Progression from CKD to end-stage renal disease (ESRD) requiring renal replacement therapy develops in 3-10% of lung transplant recipients (35-37). This population is therefore relevant to investigate the effects of former smoking on the kidney, although other factors such as CNI toxicity are involved in the renal damage in this population as well.

It seems likely that cigarette smoking may induce a direct noxious effect, e.g. through induction of systemic oxidative stress that persists for years. For toxicants present in cigarette smoke to exert a direct noxious effect on the kidney, these toxicants should be able to enter the circulation and reach the kidney. Kidneys being highly metabolically active might be susceptible to the toxic compounds present in the cigarette smoking. Recently it was found that smoking induced changes in epigenetics of megakaryocytes which are the precursors of platelets can persist for more than 10 years after smoking cessation, showing that late effects of smoking can last for many years (38,39). These findings are corroborated by data of persisting increases in risk of smoking-associated conditions long after cessation of smoking. For instance, the risk of lung cancer remains increased 15-fold in men and 9-fold in women at least until ten years after smoking cessation (40-42).Cigarette smoke consists of many components. Broadly they could be classified as hydrophilic, lipophilic or amphiphilic. It is known that lipophilic cigarette smoking components reach the distant organs like liver and kidneys (43). Additionally tobacco in cigarettes are rich in heavy metals like Arsenic, Lead, Mercury or Cadmium (44,45). The toxic components in cigarette smoke can lead to DNA damage and accelerate apoptosis (23). It has indeed been demonstrated that intra-renal vascular pathology (myo-intimal hyperplasia, arteriolar hyalinosis, glomerular sclerosis) in renal biopsies of former-smokers is more prominent than in never smokers without apparent difference in renal function (46). Thus, renal damage related to prior smoking can be present in the kidney after smoking cessation without being reflected in kidney function. Therefore a lung transplantation population is of interest in terms of mechanisms by which smoking could be detrimental for the kidney. If a sympathetic nervous system mediated vaso-constrictive effect of nicotine on renal nerves would play a role in a potential association of smoking with renal disease, one would not expect a long term association of former smoking with the renal risk in these patients. If, however, the effect is mediated by other toxic effects, former smoking could be associated with renal disease in these patients. In **chapter 3** of this thesis we therefore set out to evaluate the effects of past smoking on development of CKD in a lung transplantation population. We found a dose dependent association of cigarette smoking with the development of various stages of CKD, with higher the number of pack-years of cigarette smoking, the more rapid the progression towards higher stages of CKD. The association of former cigarette smoking with the development of various stages of CKD was independent of other known renal risk factors. This observation is another plea against the theory that the detrimental effect of smoking on kidneys is through an effect of nicotine on the sympathetic nervous system, although this should be interpreted in the context of the complex pathogenesis of progressive renal function loss after lung transplantation.

Smoking, alcohol consumption and all-cause mortality

Smoking is unequivocally detrimental, while low amounts of alcohol intake are usually considered beneficial. Nevertheless, smoking and alcohol intake often go together (47). We found a positive association of smoking with alcohol intake in our study on smoking in RTR (chapter 2). Therefore we sought out to investigate the effect of alcohol intake in RTR. It appeared that little was known about alcohol consumption in RTR. The studies that were available indicated that pre-transplant alcohol addiction was associated with early graft failure, but no prospective studies had been performed on the potential association of continued alcohol consumption after transplantation and long-term outcome (48-50). In **chapter 4** of this thesis we therefore investigated the association of smoking with alcohol consumption in RTR on one hand and the influence of smoking and alcohol consumption on graft failure and all-cause mortality on the other hand. We found that smoking was positively associated with alcohol intake. We also found a beneficial effect of moderate alcohol consumption on mortality and new onset diabetes after transplantation (NODAT) in RTR, particularly if we took the detrimental effect of smoking into account. Importantly, NODAT is now being recognized as a well-established entity and receiving increasing attention in transplantation medicine(51), in particular because NODAT limits patient survival. The mechanism by which moderate alcohol induces protection is not known. Alcohol might act as a free radical scavenger and therefore anti-oxidative. Antioxidants render protection against arteriosclerosis, reduce peripheral insulin resistance and beneficial for pancreatic beta cells. Moreover, these conditions are associated with inflammation. Alcohol containing beverages for example red wine is known to contain various anti-oxidants which might improve the overall mortality by reducing inflammation. Moreover alcohol consumption is associated with "feel good feeling" which might also indirectly influence the outcome. Resveratrol present in wine is a well-known anti-oxidant and anti-aging compound, and therefore could influence the outcome in RTR. Another underlying mechanism could be by beneficial effects on lipid profile with decreased low density lipoprotein, increased high density lipoprotein or reduced oxidized low density lipoprotein (52). However, since the majority of our population used statins, it is difficult to assess an independent effect of alcohol on lipid profile, and therefore, this should be subject of future research.

Nicotine and progressive renal failure

Cigarette smoke contains many compounds. Among them nicotine is one of the most abundant and an addictive component (53). Nicotine is used in different forms to quit smoking, also by renal patients (54). Nicotine acts through nicotinic receptors (nAchR). Among the nicotinic receptors the α7nAchR is widely studied and most described. Agonists of this receptor shows anti-inflammatory properties (55-58). Nicotine is shown to be beneficial in diseases with inflammatory components like inflammatory bowel disease, multiple sclerosis and rheumatic arthritis. Acute experiments showed impact of nicotine on renal hemodynamics: exposure to 4-mg nicotine gum (about 2 mg would be absorbed) in non- smokers significantly increased (8±1 mmHg) mean arterial pressure (MAP), whereas estimated renal plasma flow (ERPF) and GFR decreased by 15±4% and 14±4% respectively. In habitual smokers the same nicotine exposure increased MAP similarly but ERPF and GFR remained unchanged. The virtual absence of renal effects of acute nicotine administration in in smokers were ascribed to dramatic 87% increase in urinary cyclic GMP after nicotine administration. Nicotine had not changed urine volume or sodium excretion in either group (59). Similar effects of 6-mg nicotine gum on BP and GFR was observed by Ritz (31). It is, however, questionable whether these effects are sustained on the longer-term and it is unclear whether this translates into effects on renal morphology and modification of processes of renal damage. To assess the effects of long term nicotine administration on the progression of renal disease, therefore, in **chapter 5** we evaluated the effects of long term oral nicotine in a rat model of kidney disease. Nicotine treatment slightly reduced UPE with amelioration of structural damage compared to non - nicotine treated control rats. Remarkably, nicotine treatment reduced glomerulosclerosis, tubulo-interstitial inflammation and fibrosis. Thus, though nicotine was thought to be the “nephrotoxic” component of cigarette smoke, our results suggest nicotine to be “renoprotective”. This might be clinically relevant, as, first, nicotine is frequently used in quit-smoking programs, and second it could be considered as an adjunct renoprotective intervention. However, before nicotine could be considered as an additional renoprotective measure in clinical practice, many hurdles have to be overcome. First of all, there is its addictive effects. Furthermore, caution should be taken in prescribing nicotine due to its potential cardiovascular side-effects. Further research is therefore necessary to assess whether nicotine without concomitant exposure to other constituents normally present in cigarette smoke is harmful from a cardiovascular perspective (60,61). However, it is tempting to speculate that other specific α-7nAchR agonists, devoid of addictive effects, could be of therapeutic use.

In the study we did not unravel the mechanism of nicotine action. There are a number of possible explanations for our findings. We found an anti-inflammatory action of nicotine – as apparent from reduced renal influx of inflammatory cells, but others have also reported action of nicotine on intra renal vessels. It might be possible that nicotine reduced trans-glomerular pressure and thereby reduced the glomerular damage. On one hand, nicotine is known to induce endothelial nitric oxide synthase (eNOS) which is vaso-dilatory in isolated perfused kidney (62) while on the other hand, it has been shown that nicotine attenuates the renal vaso-dilatory responsiveness to some vasodilator pathways, particularly when in combination with cyclosporine (63).

Therefore, a direct effect on glomerular pressure either by efferent vasodilatation, or by afferent vasoconstriction or by a combination of both cannot be excluded, in particular because UPE was slightly higher in CON groups than in nicotine treated groups. Macrophage and endothelial cells are crucial players of inflammation . Nicotine receptors have been shown on these types of cells (57,64,65). It might be that nicotine directly reduces activation of monocytes and endothelial cells, but reduced intrarenal inflammation could also be secondary to the reduction in proteinuria. Future research therefore is needed to elucidate the mechanisms of reno-protective effects of nicotine.

Nicotine and reno-vascular neointima formation

In **chapter 6** of this thesis we evaluated the role of oral nicotine on neointima formation in two different rat models of progressive kidney disease. By serendipity we observed presence of neointima in intra renal arteries of one-year old Munich-Wistar-Fromter rats. Nicotine treatment for 28 weeks reduced the amount of neointima formation. Impact of nicotine on neo-intima formation had been described in models of primary vascular damage, thus rendering credibility to our data, that are, however, the first to shown an effect of nicotine on neo-intima formation in intrarenal vessels, in the context of renal disease. Since the finding of neointima formation in this rat model for renal disease was not previously described, and the effect of nicotine on renal neointima formation was new as well, we further tested the effect of nicotine in an experimental model of renal transplantation. In both the rat models we found that nicotine treatment reduced neointima formation. The second model, being a transplantation model, is devoid of any innervation. Our data therefore suggest that intact innervation is not necessary for nicotine mediated effects on neointima formation. There are a number of possible ways by which nicotine could modulate neointima formation. Nicotinic receptors are present on the endothelial cells as well as the macrophages (57,64,65). Both cells types play crucial roles in neointima progression. Nicotine action via such cells might slow down the progression of neointima formation. However, other cellular components might also play important roles in the process of neointima formation. For example, vascular smooth muscle cells present in the medial layer of vessels are also important for neointimal growth. It has been shown that vascular smooth muscle cells migrate to the sub-endothelial space and achieve a myofibroblast like phenotype and produce excessive extra-cellular matrix present in the neointima. Nicotine might interfere with migration, proliferation or apoptosis of smooth muscle cells. Nicotine is a known pro-angiogenic factor (66). In addition to angiogenesis, nicotine increases expression of matrix metalloproteinases (67) and in such way may potentially ameliorate some forms of neointima formation. Additionally various cytokines secreted from endothelial cells and macrophages are involved in the process of neointima progression. Nicotine might also interfere with synthesis and secretion of such cytokines therefore reducing the downstream actions on vascular smooth muscle cells. However further research is required to elucidate the mechanisms involved.

Chapter 7

Future perspectives

In this thesis we described that cigarette smoking is associated with rapid decline in kidney function in transplantation populations and that the effects persist long after quitting smoking. We furthermore found indications in an animal experimental model that nicotine is renoprotective.

Cigarette smoke contains >100 chemical compounds and many of them are toxic (68-70). Based on their affinity to water or lipid they could be either hydrophilic (lipophobic) or hydrophobic (lipophilic) or amphiphilic. Animal experiments to investigate harmful components present in cigarette smoke could be carried out with the help of smoking machine. Such experiments will generate knowledge about pathological components present in cigarette smoke, and will potentially lead to more knowledge about environmental nephrotoxicants (heavy metals, for example) and mechanisms of kidney damage. After being exposed to cigarette smoke and different subclasses of smoke components, kidneys could be analyzed for presence of different cigarette smoking components. Furthermore deposition of cigarette components could be analyzed in separate kidney compartments using staining or high performance liquid chromatography (HPLC). Additionally *in-vitro* experiments could be carried out with these components of cigarette smoke on proximal tubular epithelial cells and glomerular endothelial cells. Of note, oral nicotine had renoprotective effects, rather than deleterious effects. This is an encouraging finding taking into consideration that nicotine is often used in quit smoking programs. Accordingly, our finding could also be translated to human studies with renal vascular function measurements in subjects following a quit smoking program with oral nicotine, for example.

The mechanism of action of nicotine is not yet clear. We found that nicotine is protective in rat models of kidney disease. This effect could be because of cholinergic modulation of renal innervation, and/or via down-modulation of activation of non-neuronal cells such as endothelial cells and macrophages which are also inherently present in kidneys. To evaluate role of cholinergic nervous system on nicotine mediated renal effects further future experiment should be carried out with animals which are selectively devoid of renal innervation. *In-vitro* experiments could be carried out to investigate effects of nicotine on proliferation, apoptosis and migration of vascular smooth muscle cells and of endothelial cells. Additionally renal specific knock-out (KO) animal models of specific nAchRs should be created. Further studies with such KO models will be useful in describing roles of different nAchRs in kidney in association with nicotine. The receptors could specifically be targeted as a potential therapy for treatment of kidney diseases.

Taken together the previous studies and the studies presented in this thesis demonstrate that the renal effects of nicotine are dissociated from those of smoking, with an overall beneficial effect of nicotine as opposed to the deleterious effects of smoking. This provides interesting perspective for future research to evaluate potential the beneficial effects of nicotine in kidney disease patients.

Chapter 7

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Nederlandse Samenvatting

In de in dit proefschrift gepresenteerde studies, wordt het effect van roken en nicotine op de nier bestudeerd. Diabetes, hypertensie en overgewicht zijn belangrijke risicofactoren voor cardiovasculaire en renale ziekten. Ook is er een sterke relatie tussen leefstijlfactoren en het optreden van nierziekten. Steeds meer studies richten zich op de rol van o.a. zoutinname, lichamelijke activiteit, slaap en psychosociale stress op de progressie van chronische nierziekten. Ondanks de ontwikkeling van nieuwe therapieën en verbeterde behandelingsmogelijkheden voor patiënten, bereiken nog steeds veel patiënten het eindstadium van nierziekten, waarbij patiënten aangewezen zijn op dialyse en een eventuele niertransplantatie. Om nieuwe preventieve strategieën te ontwikkelen, is het belangrijk dat er nieuwe behandelbare risicofactoren gevonden worden. Roken is één van de risicofactoren die sterk geassocieerd is met cardiovasculaire ziekten en met de progressie van verschillende nierziekten, zoals diabetische nefropathie, IgA nefropathie, hypertensieve nierziekten etc. In dit proefschrift hebben we de rol van roken op lichaamseigen en getransplanteerde nieren onderzocht.

Roken is geïdentificeerd als een riscofactor voor de ontwikkeling en progressie van chronische nierziekten. Het roken van sigaretten kan zorgen voor nierschade door het optreden van renale vasoconstrictie, wat een afname van de renale doorbloeding bewerkstelligt; door toxische effecten van oxidatieve componenten en door blootstelling aan zware metalen. In **hoofstuk 2** van dit proefschrift hebben we de relatie tussen het roken van sigaretten op de afname van nierfunctie en het optreden van afstoting van nieren na niertransplantatie onderzocht. Hierbij vonden we dat de nierfunctie van rokers sneller afneemt dan die van niet-rokers en patiënten die gestopt zijn met roken. Er was geen verschil in de afname van de nierfunctie tussen niet-rokers en patiënten die gestopt zijn met roken. In lijn met deze resultaten vonden we een verhoogd risico op afstoting van de nier na een niertransplantatie bij huidige rokers, in vergelijking met de niet-rokers en de patiënten die gestopt zijn met roken.

Aangezien roken ook een belangrijke factor is in de progressie van chronische longziekten, hebben veel patiënten (ongeveer 60%) die een longtransplantatie ondergaan vroeger veel gerookt. Om in aanmerking te komen voor een longtransplantatie is stoppen met roken een voorwaarde. Daarom hebben we in **hoofdstuk 3** in deze populatie het effect van het vroegere rookgedrag op de ontwikkeling van chronische nierziekten onderzocht. Hierbij vonden we dat hoe meer patiënten in het verleden gerookt hadden, hoe sneller de progressie van chronische nierziekten plaatsvond. Het verband tussen het vroegere rookgedrag en de progressie van chronische nierziekten was onafhankelijk van andere bekende renale risicofactoren. De observatie dat het vroegere rookgedrag invloed heeft op de progressie van chronische nierziekten, pleit tegen de theorie dat de desastreuze gevolgen van roken op de nierfunctie optreden door het effect van nicotine op het sympathisch zenuwstelsel. Dit moet echter wel in de context van de complexe pathogenese van nierfalen na longtransplantatie gezien worden.

Hoewel de desastreuze effecten van roken onbetwist zijn, wordt de inname van kleine hoeveelheden alcohol in het algemeen als gezond gezien. Desalniettemin gaan roken en de inname van alcohol vaak samen. In **hoofdstuk 4** van dit proefschrift hebben we daarom het verband tussen roken en alcoholconsumptie onderzocht in patiënten die een niertransplantatie ondergaan. Vervolgens hebben we gekeken naar het verband tussen roken en alcoholconsumptie op afstoting van nieren na de niertransplantatie en mortaliteit. In deze patiëntenpopulatie vonden we dat roken geassocieerd is met de inname van alcohol. Matige alcoholconsumptie bleek bij deze patiënten een positief effect te hebben op de mortaliteit en het ontstaan van diabetes na transplantatie. Dit beschermende effect kwam het sterkst tot uiting wanneer de negatieve effecten van het roken in de analyse werden meegenomen. Het ontstaan van diabetes na transplantatie wordt in de transplantatiegeneeskunde tegenwoordig gezien als een belangrijk verschijnsel, aangezien het optreden hiervan de overleving van patiënten negatief beïnvloed.

Cigarettenrook bevat vele verschillende stoffen. Hiervan is nicotine een van de meest voorkomende en verslavende componenten. Nicotine wordt in verschillende modaliteiten toegepast om te stoppen met roken, ook bij nierpatiënten. Nicotine werkt via de zgn. nicotine-receptoren (nAchR), waarvan de α7nAchR de meest bekende en meest bestudeerde is. Agonisten van deze receptor zijn anti-inflammatoir. Nicotine laat inderdaad gunstige effecten zijn in ontstekingsziekten zoals inflammatory bowel disease (IBD), multiple sclerose en Rheumatoide arthritis. In **hoofdstuk 5** bestudeerden wij de effecten van chronische nicotine behandeling op de progressie van nierfalen in een rattenmodel. Nicotine behandeling leidde tot een geringe verlaging van de proteinuria en vermindering van de nierschade in vergelijking met de onbehandelde controle ratten. Met name glomerulosclerose, interstitiële ontsteking en fibrose bleken zich minder ontwikkeld to hebben in nicotine behandelde ratten. Dus, alhoewel er allerlei nefrotoxische eigenschappen werden toegedicht aan nicotine, bleek het in dit rattenmodel nierbeschermend te werken. Dit is mogelijk klinisch relevant omdat het nu naast een stop-roken middel, ook een mogelijk medicament is om nierschade te beperken.

In **hoofdstuk 6** beschrijven wij de effecten van chronische nicotine behandeling op de neointima vorming in nierslagaders in twee rattenmodellen van nierfalen. Bij toeval ontdekten wij de aanwezigheid van een neointima in nierslagaders van één jaar oude Münich-Wistar-Fromter ratten. Nicotine behandeling gedurende 28 weken kon deze neointima vorming grotendeels voorkomen. Alhoewel overeenkomstige gunstige effecten van nicotine op neointima vorming bij primaire vaatschade al langer bekend waren, zijn wij de eersten die dit nu ook aantonen in nierslagaders in de context van nierfalen. Om deze bevinding meer waarde te geven besloten wij nicotine behandeling te starten in een rattenmodel van niertransplantatie, omdat dit het belangrijkste ziektebeeld is waar in de nefrologische kliniek neointima vorming in nierslagaders voorkomt. Ook in het rattenmodel van niertransplantatie bleek nicotine de neointima vorming in nierslagaders grotendeels te kunnen voorkomen. Omdat bij niertransplantatie alle zenuwen naar en van de nier zijn doorgesneden, maar het nicotine effect ook hier werd wargenomen, duidt dit erop dat het nicotine effect op de neointima vorming niet verloopt via effecten op het perifere zenuwstelsel, maar via een direct werking op de nier. Meer onderzoek is nodig om het beschermende werkingmechanisme van nicotine op de nierslagaders te ontrafelen.

Samenvattend laten de studies in dit proefschrift nierbeschermende effect zien van chronische nicotine behandeling, in tegenstelling tot nierbeschadigende effecten van roken. Deze bevindingen openen interessante perspectieven voor toekomstig onderzoek naar de beschermende werking van nicotine bij nierpatiënten.

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